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<p>(21) International Application Number: PCT/US99/30393</p> <p>(22) International Filing Date: 10 December 1999 (10.12.99)</p> <p>(30) Priority Data: 60/111,793 11 December 1998 (11.12.98) US 60/119,312 8 February 1999 (08.02.99) US</p> <p>(71) Applicant (<i>for all designated States except US</i>): COULTER PHARMACEUTICAL, INC. [US/US]; 600 Gateway Boulevard, South San Francisco, CA 94080-7014 (US).</p> <p>(72) Inventors; and</p> <p>(75) Inventors/Applicants (<i>for US only</i>): LOBL, Thomas, J. [US/US]; 620 Plymouth Lane, Foster City, CA 94404 (US). DUBOIS, Vincent [BE/BE]; Rue De Wagenies 124, Fleurus (BE). FERNANDEZ, Anne-Marie [FR/BE]; Avenue de La Couronne 1E, B-1050 Brussels (BE). GANGWAR, Sanjeev [IN/US]; Apartment 102, 1354 Third Street, Alameda, CA 94501 (US). LEWIS, Evan [US/US]; 166 Palisades Drive, Daly City, CA 94015 (US). NIEDER, Matthew, H. [US/US]; 1107 Edgehill Drive, Burlingame, CA 94010 (US). TROUET, André [BE/BE]; Predikherengerg #29, B-3009 Herent (BE). VISKI, Peter [US/US]; 360 Talbot Avenue, Pacifica, CA 94044 (US). YARRANTON,</p>		<p>Geoffrey, T. [US/US]; 1148 Balboa Avenue, Burlingame, CA 94010 (US).</p> <p>(74) Agents: SACHDEVA, Gurjeev, K.; Cooley Godward LLP, 3000 El Camino Real, Five Palo Alto Square, Palo Alto, CA 94306-2155 (US) et al.</p> <p>(81) Designated States: AE, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CR, CU, CZ, DE, DK, DM, EE, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, TZ, UA, UG, US, UZ, VN, YU, ZA, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SL, SZ, TZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).</p> <p>Published <i>Without international search report and to be republished upon receipt of that report.</i></p>	
<p>(54) Title: PRODRUG COMPOUNDS AND PROCESS FOR PREPARATION THEREOF</p> <p>(57) Abstract</p> <p>The prodrug of the invention is a modified form of a therapeutic agent and comprises a therapeutic agent, an oligopeptide, a stabilizing group and, optionally, a linker group. The prodrug is cleavable by the enzyme truase. Also disclosed are processes for making the prodrug compounds.</p>			

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PRODRUG COMPOUNDS AND PROCESS FOR PREPARATION THEREOF

5 CROSS-REFERENCE TO RELATED APPLICATIONS

This application claims priority from U.S. Serial No. 60/111,793 filed December 11, 1998 and U.S. Serial No. 60/119,312 filed February 8, 1999.

INTRODUCTION

10 Technical Field

The present invention is directed to new compounds, and methods for making them. The compounds generally work as prodrugs and in most cases are modified versions of existing compounds or cytotoxic agents. These prodrugs have higher specificity for the intended targets and reduced specificity to unintended targets.

15

Background

Many therapeutic agents, such as anthracyclines and vinca alkaloids, and are especially effective for the treatment of cancers. However, these molecules are often characterized *in vivo* by an acute toxicity, especially a bone marrow and mucosal 20 toxicity, as well as a chronic cardiac toxicity in the case of the anthracyclines and chronic neurological toxicity in the case of the vinca alkaloids. Similarly, methotrexate may be used for the treatment of inflammatory reactions, such as rheumatic diseases, but its high toxicity limits its applications. Development of more specific antitumor agents is desirable for greater effectiveness against tumor cells and 25 a decrease in the number and severity of the side effects of these products (toxicity, destruction of non-tumor cells, etc.). Development of more specific anti-inflammatory agents is also desirable.

In order to minimize toxicity problems, therapeutic agents are advantageously presented to patients in the form of prodrugs. Prodrugs are molecules capable of 30 being converted to drugs (active therapeutic compounds) *in vivo* by certain chemical or enzymatic modifications of their structure. For purposes of reducing toxicity, this conversion should be confined to the site of action or target tissue rather than the circulatory system or non-target tissue. Prodrugs are often characterized by a low

stability in blood and serum, however, since blood and serum contain enzymes which degrade the prodrugs.

A desirable class of prodrugs that overcomes such problems have been disclosed in Patent Cooperation Treaty International Publication No. WO 96/05863

5 and in U.S. Patent No. 5,962,216, both incorporated herein by reference. Further useful prodrug compounds and methods of making such prodrugs are desirable, however, as are methods of making the prodrugs.

A particular object of the invention is a prodrug that displays a high specificity of action, a reduced toxicity, and an improved stability in blood relative to produgs of 10 similar structure (especially the closest structure) that have existed in the public domain.

SUMMARY OF THE INVENTION

The compound of the invention is a prodrug form of a therapeutic agent linked 15 directly or indirectly to an oligopeptide, which in turn, is linked to a stabilizing group.

More generally, the present invention may be described as new prodrug compounds of a therapeutic agent, especially prodrugs comprising an antitumor therapeutic agent, displaying improved therapeutic properties relative to the products of the prior art, especially improved therapeutic properties in the treatment of

20 cancerous tumors and/or in the treatment of inflammatory reactions such as rheumatic diseases. Improved therapeutic properties include decreased toxicity and increased efficacy. Particularly desired are prodrugs which display a high specificity of action, a reduced toxicity, an improved stability in the serum and blood, and which do not move into target cells until activated by a target cell associated enzyme. Prodrug 25 compounds of a marker enabling tumors to be characterized (diagnosis, progression of the tumor, assay of the factors secreted by tumor cells, etc.) are also contemplated.

The present invention also relates to the pharmaceutical composition comprising the compound according to the invention and optionally a pharmaceutically acceptable adjuvant or vehicle.

30 Further, a method of decreasing toxicity by modifying a therapeutic agent to create a prodrug is disclosed.

Several processes for creating a prodrug of the invention are described.

BRIEF DESCRIPTION OF THE DRAWINGS

Fig. 1A-1D are a table of abbreviations, names, and structures.

Fig. 2 is an exemplary scheme of cleavage of a prodrug of the invention in the extracellular vicinity of the target cell.

5 **Fig. 3** illustrates a synthesis of Fmoc- β Ala-Leu-Ala-Leu, a typical intermediate of the invention.

Fig. 4 illustrates an "Fmoc-route" synthesis of Methyl-succinyl- β Ala-Leu-Ala-Leu, a typical intermediate of the invention.

Fig. 5 illustrates an "Fmoc route" synthesis of the salt form of Suc- β Ala-Leu-Ala-Leu -DOX, a typical intermediate of the invention.

10 **Fig. 6** illustrates a "Succinyl ester route" synthesis of the salt form of Suc- β Ala-Leu-Ala-Leu -DOX, a typical intermediate of the invention.

Fig. 7 illustrates a synthesis of protected β Ala-Leu-Ala-Leu-DOX, a typical intermediate of the invention.

15 **Fig. 8** illustrates an "Allyl ester route" synthesis of the salt form of Suc- β Ala-Leu-Ala-Leu-DOX, a typical intermediate of the invention.

Fig. 9 illustrates a "Resin route" synthesis of Suc- β Ala-Leu-Ala-Leu-DOX, a typical intermediate of the invention.

Fig. 10A-10C are a table of oligopeptides useful in the prodrug of the invention.

20 **Fig. 11** is a graph of survival in a mouse xenograph model for animal given vehicle with or without drug.

Fig. 12 is a graph of survival in a mouse xenograph model comparing a doxorubicin prodrug and doxorubicin.

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DETAILED DESCRIPTION

Abbreviations

Aca = 6-Aminocaproic acid

30 ACN = Acetonitrile

Aib = Aminoisobutyric acid

All = Allyl

Aloc = Allyloxycarbonyl

Amb = 4-(Aminomethyl)benzoic acid
APP = 3-Amino-3-phenylpropionic acid
DCC = N,N' - Dicyclohexylcarbodiimide
Boc = t-butyloxycarbonyl
5 Cap = caproic acid
DBN = 1,5 Diazabicyclo [4.3.0] non-5-ene
DBO = 1,4 Diazabicyclo [2.2.2] octane
DBU = 1,8-Diazabicyclo [5.4.0] Undec-7-ene
DCM = Dichloromethane
10 DIC = N,N' - Diisopropylcarbodiimide
DIEA = Diisopropylethylamine
Dg = Diglycolic Acid
DMF = Dimethylformamide
DNR = Daunorubicin
15 Dox = Doxorubicin
Et₂O = diethyl ether
Fmoc = 9-Fluorenylmethyloxycarbonyl
Gl = Glutaric Acid
HATU = O-(7-Azabenzotrazol-1-yl)-1,1,3,3-tetramethyluronium-
20 hexafluorophosphate
HBTU = 2-(1H-Benzotriazole-1-yl)1,1,3,3-tetramethyluronium-hexafluorophosphate
HEPES - Hydroxethylpiperidine
HOBt = N-Hydroxybenzotriazole
HPLC = High pressure liquid chromatography
25 MeOH = Methanol
NAA = 3-Amino-4,4-diphenylbutyric Acid
Nal = 2-Naphthylalanine
Naph = 1,8 – Naphthalene dicarboxylic acid
Nle = Norleucine
30 NMP = N-methylpyrrolidine
Nva = Norvaline
PAM resin = 4-hydroxymethylphenylacetamidomethyl
Phg = Phenylglycine
Pyg = Pyroglutamic acid

Pyr = 3-Pyridylalanine

RT, rt = Room temperature

Suc = Succinic Acid

TCE = trichloroethyl

5 TFA = Trifluoroacetic acid

THF = Tetrahydrofuran

Thi = 2-Thienylalanine

Thz = Thiazolidine-4-carboxylic acid

Tic = Tetrahydroisoquinoline-3-carboxylic acid

10

The compound of the invention is a prodrug form of a therapeutic agent linked directly or indirectly to an oligopeptide, which in turn, is linked to a stabilizing group.

More generally, the present invention may be described as new prodrug compounds of a therapeutic agent, especially prodrugs comprising an antitumor therapeutic agent, displaying improved therapeutic properties relative to the products of the prior art, especially improved therapeutic properties in the treatment of cancerous tumors and/or in the treatment of inflammatory reactions such as rheumatic diseases. Improved therapeutic properties include decreased toxicity and increased efficacy. Particularly desired are prodrugs which display a high specificity of action, 20 a reduced toxicity, an improved stability in the serum and blood, and which do not move into target cells until activated by a target cell associated enzyme. Prodrug compounds of a marker enabling tumors to be characterized (diagnosis, progression of the tumor, assay of the factors secreted by tumor cells, etc.) are also contemplated.

The present invention also relates to the pharmaceutical composition 25 comprising the compound according to the invention and optionally a pharmaceutically acceptable adjuvant or vehicle.

Further, a method of decreasing toxicity by modifying a therapeutic agent to create a prodrug is disclosed.

Several processes for creating a prodrug of the invention are described.

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Prodrug

The prodrug of the invention is a modified form of a therapeutic agent and comprises several portions, including:

- (1) a therapeutic agent,

- (2) an oligopeptide, and
- (3) a stabilizing group, and
- (4) optionally, a linker group.

5 Each of the portions of the prodrug are discussed in greater detail below. The typical orientation of these portions of the prodrug is as follows:

(stabilizing group)-(oligopeptide)-(optional linker group)-(therapeutic agent).

10 The stabilizing group is directly linked to the oligopeptide at a first attachment site of the oligopeptide. The oligopeptide is directly or indirectly linked to the therapeutic agent at a second attachment site of the oligopeptide. If the oligopeptide and the therapeutic agent are indirectly linked, then a linker group is present.

15 Direct linkage of two portions of the prodrug means covalent binding between the two portions. The stabilizing group and the oligopeptide are therefore directly linked via covalent binding at the first attachment site of the oligopeptide, typically the N-terminus of the oligopeptide. When the oligopeptide and the therapeutic agent are directly linked then they are covalently bound to one another at the second attachment site of the oligopeptide. The second attachment site of the oligopeptide is 20 typically the C-terminus of the oligopeptide, but may be elsewhere on the oligopeptide.

25 Indirect linkage of two portions of the prodrug means each of the two portions is covalently bound to a linker group. In an alternative embodiment, the prodrug has indirect linkage of the oligopeptide to the therapeutic agent. Thus, typically, the oligopeptide is covalently bound to the linker group which, in turn, is covalently bound to the therapeutic agent.

30 The prodrug of the invention is cleavable within the oligopeptide directly or indirectly linked to the therapeutic agent. In order for the prodrug to be effective, the oligopeptide linked to the therapeutic agent is either the active portion of the prodrug itself or is readily convertible to the active portion of the prodrug usually by one or more exopeptidases. The active portion of the prodrug is that part of the prodrug which upon release from the remaining portion of the prodrug compound enter the target cell and exert the therapeutic effect directly or often further conversion within the target cell.

The structures of the stabilizing group and oligopeptide are further selected to limit clearance of the oligopeptide by enzymes other than those which may be present in blood or non-target tissue. The stabilizing group blocks degradation of the prodrug and may act in providing preferable charge or other physical prodrug characteristics 5 of the prodrug by exopeptidases. The amino acid sequence of the oligopeptide is designed to further ensure specificity for trouse.

It is desirable to make a therapeutic agent, especially an antitumor and/or anti-inflammato¹⁰ry therapeutic agent, inactive by modification of the therapeutic agent to a prodrug form. According to the invention, the target cells are usually tumor cells or cells participating in anti-inflammatory reactions, especially those associated with rheumatic diseases, such as macrophages and monocytes. Modification of the therapeutic agent to a prodrug form also tends to reduce some of the side effects of the therapeutic agents.

In the target cell, the therapeutic agent (optionally attached to one or two 15 amino acids and possibly also linker group) acts either directly on its specific intracellular action site or, after a modification under the action of intracellular proteases, kills the target cell or blocks its proliferation. Since normal cells liberate little to no trouse *in vivo*, the compound according to the invention is maintained inactive and does not enter the normal cells or does so in a relatively minor amount.

20 The prodrug is administered to the patient, carried through the blood stream in a stable form, and when in the vicinity of a target cell, is acted upon by trouse. Since the enzyme is only minimally present within the extracellular vicinity of normal cells, the prodrug is maintained and its active portion (including the therapeutic agent) and gains entry into the normal cells only minimally, at best. In the vicinity of tumor or 25 other target cells, however, the presence of the relevant enzyme in the local environment causes cleavage of the prodrug. The example shown in the Fig. 2 depicts N-capped an tetrapeptide prodrug being cleaved from the remainder of the prodrug extracellularly and gaining entry into the target cell. Once within the target cell, it may be further modified to provide therapeutic effect. While the active portion of the 30 prodrug may also enter the normal cells to some extent, the active portion is freed from the remainder of the prodrug primarily in the vicinity of target cells. Thus, toxicity to normal cells is minimized.

Release of the active portion of the prodrug including the therapeutic agent preferably occurs in the immediate environment of the target cell. In the target cell,

the therapeutic agent acts either directly on its specific intracellular action site or, after a modification under the action of intracellular proteases or other enzymes, it may be modified to another form in which kills the target cell or blocks its proliferation. A schematic diagram of this action for an exemplary prodrug of the invention is shown 5 in Fig. 2.

This process is particularly useful for, and is designed for, target cell destruction when the target tissue excretes an enzyme or other factor that is not secreted by normal cells. Here "normal cells" means non-target cells that would be encountered by the prodrug upon administration of the prodrug in the manner 10 appropriate for its intended use. Since normal (i.e., non-target) cells liberate little or none of the target-cell enzyme(s) that are responsible for cleaving the bond that links the active portion (including the therapeutic agent) of the prodrug from the remainder of the prodrug *in vivo*, the compound of the invention is maintained inactive and does not enter the normal cells.

15 In an alternative embodiment, the orientation of the prodrug may be reversed so that a C terminus block of the oligopeptide is attached to the oligopeptide and the therapeutic agent is directly or indirectly linked to the N terminus of the oligopeptide.

Trouase

20 Trouase is the enzyme which is thought to be critical for specific activation of prodrug at the target tissue. Trouase is an endopeptidase which shows a remarkable degree of discrimination between leucine and isoleucine at the carboxyl side of the oligopeptide cleavage site. A defining characteristic is that under appropriate assay conditions, trouase readily cleaves succinyl- β AlaLeuAlaLeu-Daunorubicin while it is 25 at least twenty-fold less active with succinyl- β AlaIleAlaLeu-Daunorubicin.

Trouase is believed to be associated with target cells. Most likely it is generated either by target cells or by normal cells that are associated with the target cells, such as stromal tissue or macrophages. So, for example, the trouase may be secreted or present in some other manner in the extracellular vicinity of the target cell.

30 In many cases, the prodrug of the invention includes a therapeutic agent for the treatment of cancer and the target cell is a tumor cell. Thus, trouase may be secreted extracellularly by the target cell or it may be present extracellularly because there is a

fair amount of cell lysis associated with tumors generally. Cell lysis is also associated with inflammatory tissue, another target site.

Trouase activity is low in human plasma, however. Trouase activity has been observed in carcinoma cell extracts and conditioned media from cultured carcinoma 5 cells, red blood cells and various human tissues, especially kidney. Carcinoma cell trouase has an apparent pI of ~5.1, a molecular weight by gel filtration of about 68 kD and a neutral pH activity optimum. It is inhibited by the metalloproteinase inhibitors EDTA and 1,10-phenanthroline but not serine, thiol, or acid proteinase inhibitors such as aminoethylbenzene-sulfonate, E64, pepstatin, leupeptin aprotinin, CA074, or 10 fumagillin. Furthermore EDTA inactivated trouase can be re-activated by cobalt (50-100 μ M) and manganese (50-1000 μ M) but not zinc or cupric cations.

A partially purification scheme of trouase from HeLa cervical carcinoma cell homogenate ultracentrifugation (145,000xg 30 min) supernatant consists of four steps as follows:

- 15 1. Anion exchange chromatography using a 15Q column (*Pharmacia*) eluted with a 0 to 0.5 M NaCl linear gradient in 20 mM triethylamine chloride pH 7.2, 0.01% Triton X-100,
2. Affinity chromatography using Chelating Sepharose Fast Flow (*Pharmacia*) pre-loaded with CoCl₂ and eluted with a 0 to 100 mM imidazole linear gradient in 10 mM sodium phosphate, 0.5 M NaCl, pH 7.2, 0.01% Triton X-100, 0.02% NaN₃.
- 20 3. Preparative native electrophoresis
4. Gel filtration high performance liquid chromatography using a 7.8 mm X 60 cm TSK Gel G-3000SWXL (TosoHaas) eluted with 0.3 mL/min 50 mM potassium phosphate, 200 mM potassium sulfate, pH 7.2.

Further cleavage of the active portion of the prodrug after trouase cleavage 30 may occur intracellularly or extracellularly and is believed to be catalyzed by amino-exopeptidases. In vitro experiments indicate that amino-exopeptidases of broad specificities are present in human blood as well as the carcinoma cell environment.

Stabilizing Group

An important portion of the prodrug is the stabilizing group, which serves to protect the prodrug compound from degradation in circulating blood when it is administered to the patient and allows the prodrug to reach the vicinity of the target cell relatively intact. The stabilizing group protects the prodrug from degradation by 5 proteinases and peptidases present in blood, blood serum, and normal tissue. Particularly, since the stabilizing group caps the N-terminus oligopeptides, and is therefore sometimes referred to as an N-cap or N-block, it serves to ward against exopeptidases to which the prodrug may otherwise be susceptible.

10 The compound is less toxic *in vivo* than the starting therapeutic agent because the prodrug is not cleaved in blood, heart, brain, bone marrow, in the mucosa and the like. This decrease in toxicity applies, in particular, to the acute effects such as marrow and mucosal toxicity, as well as possible cardiac or neurological toxicity.

15 Ideally, the stabilizing group is useful in the prodrug of the invention if it serves to protect the prodrug from degradation, especially hydrolysis, when tested by storage of the prodrug compound in human blood at 37°C for 2 hours and results in less than 20%, preferably less than 2%, cleavage of the prodrug by the enzymes present in the human blood under the given assay conditions.

20 More particularly, the stabilizing group is either
(1) a non-amino acid, or
(2) an amino acid that is either (i) a non-genetically-encoded amino acid having four or more carbons or (ii) aspartic acid or glutamic acid attached to the N-terminus of the oligopeptide at the β -carboxy group of aspartic acid or the γ -carboxyl group of glutamic acid.

25 For example, dicarboxylic (or a higher order carboxylic) acid or a pharmaceutically acceptable salt thereof may be used as a stabilizing group. A preferred list of non-amino acid stabilizing groups is Succinic acid, Diglycolic acid, Maleic acid, Polyethylene glycol, Pyroglutamic acid, Acetic acid, 1 or 2 Naphthylcarboxylic acid and Glutaric acid.

30 Additionally, intravascular administration of an aggregating positively charged prodrug in mice resulted in acute toxicity. However, no such toxicity was observed when the charge on this prodrug was reversed by derivitization with a negatively charged stabilizing group. This effect is discussed in greater detail below.

Thus, where aggregation of the therapeutic agent is a concern, it is preferred that the linked stabilizing group be negatively charged or neutral.

In vivo Toxicity

5 Many cytotoxic compounds have inherent low solubility. Positively charged anthracyclines for example form aggregates at high concentration and these aggregates induce intravenous coagulation when the aggregates are administered iv. We have discovered that trouse recognizes a specific set of hydrophobic peptide sequences. When one of these hydrophobic sequences (e.g., β -Ala-Leu-Ala-Leu) is 10 conjugated to a cytotoxic compound (for example: Doxorubicin), it results in a less soluble compound which forms large aggregates when in aqueous formulations for iv injection (the preferred method of delivery of anti-cancer drugs). Since most peptides have exposed, positively charged amino termini at physiological pH, these aggregates form a polypositively charged surface *in vivo*. These aggregates given iv induced a 15 coagulation cascade and death in mice within a few minutes (usually less than 30 min) of administration. This renders any positively charged prodrugs that are formulated with aggregate suspension unsuitable for use.

Several experiments support the hypothesis aggregates are formed with peptide conjugated Doxorubicins. The examination of similarly formulated solutions 20 by laser light scattering and size exclusion ultrafiltration demonstrated that only a small amount of the material had a molecular weight below 10 kD. The average molecular size of the aggregates were found to be around 70 kD. When the animals were concomitantly administered (see example 6) heparin with the iv dose the acute toxicity was greatly reduced or eliminated. When the animals were given dilute 25 solutions of the same drug (same total dose) there was no acute toxicity. These results taken together with the literature reports support the conclusion that peptide prodrugs of compounds that form aggregates because of insufficient solubility do not make optimal therapeutics. A solution to this aggregate problem makes these peptide prodrugs more practical. When these peptide prodrugs form aggregates because of 30 insufficient solubility at the desired formulated concentrations, stabilizing group on the peptide chain must terminate in a negatively charged or a neutral functionality. The use of succinyl as a stabilizing group on the peptide prodrug renders the prodrug

not acutely toxic (see example 6). This solves an important problem in the use of peptide prodrugs as practical therapies for humans.

Since chemical radicals having more than two carboxylic acids are also acceptable as part of the prodrug, the end group having the dicarboxylic (or higher order carboxylic) acids is more generally defined as N-cap. N-cap as used herein is a monoamide derivative of a chemical radical containing two or more carboxylic acids where the amide is attached onto the amino terminus of the peptide and the remaining carboxylic acids are free and uncoupled. For this purpose, the N-cap is preferably succinic acid, glutaric acid, or phthalic acid, with succinic acid being most preferred.

10 Other examples of useful N-caps in the prodrug compound of the invention include diglycolic acid, fumaric acid, naphthalene dicarboxylic acid, aconitic acid, carboxycinnamic acid, triazole dicarboxylic acid, gluconic acid, 4-carboxyphenyl boronic acid, polyethylene glycolic acid, butane disulfonic acid, and maleic acid.

15

Oligopeptide

Oligopeptides are generally defined as polypeptides of short length, typically twenty amino acids or fewer. An oligopeptide useful in the prodrug of the invention is of at least four amino acids in length, however. At the upper end, oligopeptides of 20 less than or equal to twelve amino acids are most useful, although an oligopeptide may have a chain length greater than twelve amino acids and fall within both the definition of the term as generally recognized in the scientific field and additionally within the scope of the invention. Thus, the oligopeptide portion of the prodrug of the invention has four or more amino acids. Typically, the oligopeptide portion of the 25 prodrug of the invention has four to twelve amino acids, inclusive.

Numbering Scheme

The oligopeptide has a formula or sequence $(AA)_n-AA^4-AA^3-AA^2-AA^1$, wherein:

30 each AA independently represents any genetically encoded amino acid;
n is an integer from 0 to 12;
 AA^4 represents a non-genetically-encoded amino acid;
 AA^3 represents any amino acid;
 AA^2 represents any amino acid; and

AA¹ represents any amino acid.

This corresponds to a position sequence P(n-2)-P2-P1-P1'-P2'.

The trouse is believed to cleave between the P1 and P1' positions.

Unless otherwise indicated, all amino acids are in the L configuration.

5

Preferred amino acids in the oligopeptide are as follows:

In the P2 position, one of the following: β -Alanine, Thiazolidine-4-carboxylic acid, 2-

Thienylalanine, 2-Naphthylalanine, D-Alanine, D-Leucine, D-Methionine, D-

10 Phenylalanine, 3-Amino-3-phenylpropionic acid, γ -Aminobutyric acid, 3-amino-4,4-diphenylbutyric acid.

Also possible are Tetrahydroisoquinoline-3-carboxylic acid, 4-Aminomethylbenzoic acid Aminoisobutyric acid in the P2 position.

15

In the P1 position, one of the following: Leucine, Tyrosine, Phenylalanine, *p*-Cl-Phenylalanine, *p*-Nitrophenylalanine, Valine, Norleucine, Norvaline, Phenylglycine, Tryptophan, Tetrahydroisoquinoline-3-carboxylic acid, 3-Pyridylalanine, Alanine, Glycine, Thienylalanine.

20

Also possible are Methionine, Valine, Proline in the P1 position.

In the P1' position, one of the following: Alanine, Leucine, Tyrosine, Glycine, Serine, 3-Pyridylalanine, 2-Thienylalanine.

25

Also possible are Aminoisobutyric Acid, Threonine, Phenylalanine

In the P2' position, one of the following: Leucine, Phenylalanine, Isoleucine, Alanine, Glycine, Tyrosine, 2-Naphthylalanine, Serine.

30

Also possible is β -Alanine in the P2' position.

Oligopeptides useful in the prodrug of the invention include the following: D-AlaThi β Ala β AlaLeuAlaLeu (SEQ ID NO: 1), Thi β Ala β AlaLeuAlaLeu (SEQ ID NO:

2). β Ala β AlaLeuAlaLeu (SEQ ID NO: 3), β AlaAlaAlaIle (SEQ ID NO: 4),
 β AlaAlaAlaLeu (SEQ ID NO: 5), β AlaPheTyrLeu (SEQ ID NO: 6), β AlaPheThrPhe
(SEQ ID NO: 7), β AlaPheGlyIle (SEQ ID NO: 8), β AlaPheGlyLeu (SEQ ID NO: 9),
 β AlaPhePhePhe (SEQ ID NO: 10), β AlaPhePheIle (SEQ ID NO: 11),
5 β AlaPhePheLeu (SEQ ID NO: 12), β AlaPheAlaIle (SEQ ID NO: 13),
 β AlaPheAlaLeu (SEQ ID NO: 14), ThiGlyAlaLeu (SEQ ID NO: 15), NalGlyAlaLeu
(SEQ ID NO: 16), β AlaLeuTyrLeu (SEQ ID NO: 17), β AlaLeuThiLeu (SEQ ID NO:
18), β AlaLeuThrPhe (SEQ ID NO: 19), β AlaLeuThrIle (SEQ ID NO: 20),
 β AlaLeuThrLeu (SEQ ID NO: 21), β AlaLeu (SerLeu (SEQ ID NO: 22),
10 β AlaLeuPyrLeu (SEQ ID NO: 23), β AlaLeuLeuLeu (SEQ ID NO: 24),
 β AlaLeuGlyPhe (SEQ ID NO: 25), β AlaLeuGlyIle (SEQ ID NO: 26), ThiLeuGlyLeu
(SEQ ID NO: 27), β AlaLeuGlyLeu (SEQ ID NO: 28), AibLeuGlyLeu (SEQ ID NO:
29), β AlaLeuPhelle (SEQ ID NO: 30), β AlaLeuPheLeu (SEQ ID NO: 31),
 β AlaLeuAibLeu (SEQ ID NO: 32), β AlaLeuAlaAla (SEQ ID NO: 33),
15 β AlaLeuAla β Ala (SEQ ID NO: 34), β AlaLeuAlaPhe (SEQ ID NO: 35),
 β AlaLeuAlaGly (SEQ ID NO: 36), β AlaLeuAlaIle (SEQ ID NO: 37),
 β AlaLeuAlaLeu (SEQ ID NO: 38), TicLeuAlaLeu (SEQ ID NO: 39), ThzLeuAlaLeu
(SEQ ID NO: 40), ThiLeuAlaLeu (SEQ ID NO: 41), NalLeuAlaLeu (SEQ ID NO:
42), NAALeuAlaLeu (SEQ ID NO: 43), D-LeuLeuAlaLeu (SEQ ID NO: 44), D-
20 AlaLeuAlaLeu (SEQ ID NO: 45), D-MetLeuAlaLeu (SEQ ID NO: 46),
APPLeuAlaLeu (SEQ ID NO: 47), AmbLeuAlaLeu (SEQ ID NO: 48),
 β AlaLeuAlaNal (SEQ ID NO: 49), β AlaLeuAla (Ser (SEQ ID NO: 50),
 β AlaLeuAlaTyr (SEQ ID NO: 51), β AlaMetTyrPhe (SEQ ID NO: 52),
 β AlaMetTyrLeu (SEQ ID NO: 53), β AlaMetGlyIle (SEQ ID NO: 54), ThiMetGlyLeu
25 (SEQ ID NO: 55), β AlaMetPhePhe (SEQ ID NO: 56), β AlaMetPhelle (SEQ ID NO:
57), TicMetAlaLeu (SEQ ID NO: 58), NalMetAlaLeu (SEQ ID NO: 59),
NAAMetAlaLeu (SEQ ID NO: 60), β AlaMetAlaLeu (SEQ ID NO: 61),
APPMetAlaLeu (SEQ ID NO: 62), β AlaNleTyrIle (SEQ ID NO: 63), β AlaNleTyrLeu
(SEQ ID NO: 64), β AlaNleThrIle (SEQ ID NO: 65), β AlaNleThrLeu (SEQ ID NO:
30 66), β AlaNleGlyPhe (SEQ ID NO: 67), β AlaNleGlyIle (SEQ ID NO: 68),
 β AlaNleGlyLeu (SEQ ID NO: 69), β AlaNlePhelle (SEQ ID NO: 70), β AlaNleAlaIle
(SEQ ID NO: 71), β AlaNleAlaLeu (SEQ ID NO: 72), β AlaNleAlaPhe (SEQ ID NO:

73). β AlaNvaAlaLeu (SEQ ID NO: 74), β AlaPheTyrIle (SEQ ID NO: 75),
ThiProGlyLeu (SEQ ID NO: 76), ThiProAlaLeu (SEQ ID NO: 77), NalProAlaLeu
(SEQ ID NO: 78), β AlaProAlaLeu (SEQ ID NO: 79), β AlaPhe(Cl)AlaLeu (SEQ ID
NO: 80), β AlaPhe(NO₂)AlaIle (SEQ ID NO: 81), β AlaPhe(NO₂)AlaLeu (SEQ ID
NO: 82), β AlaPhgAlaLeu (SEQ ID NO: 83), β AlaPyrAlaLeu (SEQ ID NO: 84),
TicThrGlyLeu (SEQ ID NO: 85), β AlaThiGlyIle (SEQ ID NO: 86), β AlaThiAlaLeu
(SEQ ID NO: 87), β AlaTicAlaIle (SEQ ID NO: 88), β AlaTicAlaLeu (SEQ ID NO:
89), β AlaValAlaLeu (SEQ ID NO: 90), β AlaTrpAlaLeu (SEQ ID NO: 91),
 β AlaTyrTyrPhe (SEQ ID NO: 92), β AlaTyrTyrIle (SEQ ID NO: 93), β AlaTyrTyrLeu
10 (SEQ ID NO: 94), β AlaTyrThrLeu (SEQ ID NO: 95), β AlaTyrPheLeu (SEQ ID NO:
96), β AlaTyrGlyIle (SEQ ID NO: 97), ThiTyrGlyLeu (SEQ ID NO: 98),
 β AlaTyrGlyLeu (SEQ ID NO: 99), β AlaTyrPhelle (SEQ ID NO: 100), β AlaTyrAlaIle
(SEQ ID NO: 101), ThiTyrAlaLeu (SEQ ID NO: 102), and β AlaTyrAlaLeu (SEQ ID
NO: 103).

15

Blocking Amino Acid

The oligopeptide portion of the prodrug includes a blocking amino acid as
AA⁴ of the oligopeptide sequence, i.e. at position P2 of the position sequence,
according to the numbering scheme described above. The blocking amino acid is a
20 non-genetically-encoded amino acid.

The function of the blocking amino acid at position P2 is to maintain
selectivity for cleavage of the prodrug by trouse and inhibit cleavage of the
oligopeptide by other enzymes in that portion of the oligopeptide most closely linked
(directly linked or indirectly linked) to the therapeutic agent portion of the prodrug
25 compound. More particularly, by placing a blocking amino acid at position P2,
undesirable cleavage within the peptide linkages of the four amino acids of the
oligopeptide sequence AA⁴-AA³-AA²-AA¹ and position sequence P2-P1-P1'-P2' is
reduced. It is believed that trouse cleaves between the P1 and P1' positions of the
oligopeptide. Since it is known that blood and normal cells are associated with a
30 variety of peptidases, placing a blocking amino acid at position P2 serves to protect
the oligopeptide portion of the prodrug *in vivo* until the prodrug is in the vicinity of
the target cell. Specifically, by placing a blocking amino acid at position P2, it is
believed that the oligopeptide is protected from undesirable cleavage between P2 and

P1. Without the blocking amino acid, the prodrug might be vulnerable to both exopeptidases and endopeptidases present in blood and normal tissue, both classes of enzymes which might otherwise degrade the prodrug before it reaches its target.

Example 2 below illustrates this important feature of the prodrug.

5

Therapeutic Agents

Therapeutic agents that are particularly useful for modification to a prodrug form according to the invention are those with narrow therapeutic window. A drug or therapeutic agent with a narrow therapeutic window is one which the dose at which 10 toxicity is evident, by general medical standards, is too close to the dose at which efficacy is evident.

The therapeutic agent conjugated to the stabilizing group and oligopeptide and, optionally, the linker group to form the prodrug of the invention may be useful for treatment of cancer, inflammatory diseases, or some other medical condition.

15 Preferably, the therapeutic agent is selected from the following class of compounds: Alkylating Agents, Antiproliferative agents, Tubulin Binding agents, Vinca Alkaloids, Enediynes, Podophyllotoxins or Podophyllotoxin derivatives, the Pteridine family of drugs, Taxanes, Anthracyclines, Dolastatins, Topoisomerase inhibitors, *cis*-Platinums

20 Particularly, the therapeutic agent is advantageously selected from the following compounds: Doxorubicin, Daunorubicin, Vinblastine, Vincristine, Calicheamicin, Etoposide, Etoposide phosphate, CC-1065, Duocarmycin, KW-2189, Methotrexate, Methopterin, Aminopterin, Dichloromethotrexate, Docetaxel, Paclitaxel, Epithiolone, Combretastatin, Combretastatin A₄ Phosphate, Dolastatin 10, 25 Dolastatin 11, Dolastatin 15, Topotecan, Camptothecan, Mitomycin C, Porfiromycin, 5-Flurouracil, 6-Mercaptopurine, Fludarabine, Tamoxifen, Cytosine arabinoside, Adenosine Arabinoside, Colchicine, Carboplatin, Mitomycin C, Bleomycin, Melphalan or a derivative or analog thereof.

30

Linker Groups

A linker group between the oligopeptide and the therapeutic agent may be advantageous for reasons such as the following:

1. As a spacer for steric considerations in order to facilitate enzymatic release of the AA¹ amino acid.
2. To provide an appropriate attachment chemistry between the therapeutic agent and the oligopeptide.
- 5 3. To improve the synthetic process of making the prodrug conjugate (e.g., by pre-derivitizing the therapeutic agent or oligopeptide with the linker group before conjugation to enhance yield or specificity.)
4. To improve physical properties of the prodrug.
- 10 5. To provide an additional mechanism for intracellular release of the drug.

Linker structures are dictated by the required functionality. Examples of potential linkers chemistries are hydrazide, ester, ether, and sulphydryl. Amino caproic acid is an example of a bifunctional linker group. When amino caproic acid is used in the linker group, it is not counted as an amino acid in the numbering scheme 15 of the oligopeptide.

Pharmaceutical Compositions

The invention also includes a pharmaceutical composition comprising a compound, particularly a prodrug compound, according to the invention and, 20 optionally, a pharmaceutically acceptable adjuvant or vehicle.

The invention also relates to the use of the pharmaceutical composition for the preparation of a medicinal product intended for the treatment of a medical condition.

The pharmaceutical composition may, for example, be administered to the patient parenterally, especially intravenously, intramuscularly, or intraperitoneally. 25 Pharmaceutical compositions of the invention for parenteral administration comprise sterile, aqueous or nonaqueous solutions, suspensions, or emulsions. As a pharmaceutically acceptable solvent or vehicle, propylene glycol, polyethylene glycol, injectable organic esters, for example ethyl oleate, or cyclodextrins may be employed. These compositions can also comprise wetting, emulsifying and/or 30 dispersing agents.

The sterilization may be carried out in several ways, for example using a bacteriological filter, by incorporating sterilizing agents in the composition or by irradiation. They may also be prepared in the form of sterile solid compositions which

may be dissolved at the time of use in sterile water or any other sterile injectable medium.

The pharmaceutical composition may also comprise adjuvants which are well known in the art (e.g., vitamin C, antioxidant agents, etc.) and capable of being used 5 in combination with the compound of the invention in order to improve and prolong the treatment of the medical condition for which they are administered.

Doses for administration to a patient of the compounds according to the invention are generally at least the usual doses of the therapeutic agents known in the field, described in Bruce A. Chabner and Jerry M. Collins, Cancer Chemotherapy,

10 Lippincott Ed., ISBN 0-397-50900-6 (1990) or they may be adjusted, within the judgment of the treating physician, to accommodate the superior effectiveness of the prodrug formulations or the particular circumstances of the patient being treated. The doses administered hence vary in accordance with the therapeutic agent used for the preparation of the compound according to the invention.

15

Treatment with Prodrug Compound

A method for the therapeutic treatment of a medical condition comprising administering, especially parenterally or intravenously, to the patient a therapeutically effective dose of the pharmaceutical composition is also within the scope of the 20 invention.

The prodrug compound is useful for the treatment of many medical conditions including cancer, neoplastic diseases, tumors, inflammatory diseases, and infectious diseases. Examples of preferred diseases are breast cancer, colorectal cancer, liver 25 cancer, lung cancer, prostate cancer, ovarian cancer, brain cancer, and pancreatic cancer. Formulated in pharmaceutically acceptable vehicles (such as isotonic saline), the prodrug compound can be administered to animals or humans in intravenous doses ranging from 0.05 mg/kg/dose/day to 300 mg/kg/dose/day. It can also be administered as intravenous drip or other slow infusion method.

30 Human patients are the usual recipients of the prodrug of the invention, although veterinary usage is also contemplated.

PROCESS CHEMISTRY GENERAL PROCEDURES

Oligopeptide: General Method for the synthesis of peptides

The peptide, or oligopeptide, sequences in the prodrug conjugates of this invention may be synthesized by the solid phase peptide synthesis (using either Boc or Fmoc chemistry) methods or by solution phase synthesis. The general Boc and 5 Fmoc methods are widely used and are described in the following references: Merrifield, *J. A. Chem. Soc.*, 88:2149 (1963); Bodanszky and Bodanszky, *The Practice of Peptide Synthesis*, Springer-Verlag, Berlin, 7-161 (1994); Stewart, *Solid Phase Peptide Synthesis*, Pierce Chemical, Rockford, (1984).

10 General Fmoc Solid Phase Method

Using the preferred solid phase synthesis method, either automated or manual, a peptide of desired length and sequence is synthesized through the stepwise addition of amino acids to a growing chain which is linked to a solid resin. Examples of useful Fmoc compatible resins, but not limited to, are Wang resin, HMPA-PEGA resin, Rink 15 acid resin, or a hydroxyethyl-photolinker resin. The C-terminus of the peptide chain is covalently linked to a polymeric resin and protected α -amino amino acids were added in a stepwise manner with a coupling reagent. A preferred α -amino protecting group is the Fmoc group, which is stable to coupling conditions and can readily be removed under mild alkylne conditions. The reaction solvents are preferably but not 20 limited to DMF, NMP, DCM, MeOH, and EtOH. Examples of coupling agents are: DCC, DIC, HATU, HBTU. Cleavage of the N-terminal protecting group is accomplished in 10 - 100% piperidine in DMF at 0 - 40°C, with ambient temperature being preferred. At the end of synthesis the final Fmoc protecting group is removed using the above N-terminal cleavage procedure. The remaining peptide on resin is 25 cleaved from the resin along with any acid sensitive side chain protecting groups by treating the resin under acidic conditions. For example an acidic cleavage condition is a mixture of trifluoroacetic acid (TFA) in dichloromethane. If the hydroxyethyl-photolinker resin is used, the appropriate wavelength for inducing cleavage is λ 365 nm ultraviolet light. A diagrammatic representation of this process is given in Fig. 3.

30

General N-cap Method via Solid Phase Synthesis

The preparation of N-terminus derivatized peptides is conveniently accomplished on solid phase. When the peptide synthesis is complete and the

terminal Fmoc is removed while the peptide is still on the solid support. The N-cap of choice is coupled next using standard peptide coupling conditions onto the N-terminus of the peptide. On completion of the N-cap coupling the peptide is cleaved from the resin using the procedure described above.

5

General Boc Solid Phase Method

For the solid phase method using Boc chemistry, either the Merrifield resin or PAM resin is useful. The amino acids are coupled to the growing chain on solid phase by successive additions of coupling agent activated Boc-protected amino acids.

10 Examples of coupling agents are: DCC, DIC, HATU, HBTU. The reaction solvents may be DMF, DCM, MeOH, and NMP. Cleavage of the Boc protecting group is accomplished in 10 - 100% TFA in DCM at 0 - 40°C, with ambient temperature being preferred. On completion of the peptide chain assembly the N-terminus protecting group (usually Boc) is removed as described above. The peptide is removed from the 15 resin using liquid HF or trifluoromethane sulfonic acid in dichloromethane.

General Procedure for the Preparation of Fmoc Oligopeptide by Solution Phase Synthesis

Alternatively, the prodrug peptide intermediate may be made via a solution phase 20 synthesis, utilizing either Boc or Fmoc chemistry. In the diagrammatic presentation of the methods (Fig. 4), the C-terminal Leu tetrapeptide is generally used as an example, but it will be understood that similar reactions may be performed with other C-terminal tetrapeptides, as well. The peptide can be built up by the stepwise assembly in analogy to the solid phase method (in the N-terminal direction or in the C-terminal direction) or through the coupling of 25 two suitably protected dipeptides or a tripeptide with a single amino acid.

One method of solution phase synthesis is a stepwise building up of the prodrug peptide intermediate using Fmoc chemistry, shown in Fig. 4. The C-terminus must be protected to reduce the formation of side products. The C-terminal R group in Fig. 4 is Me, tBu, benzyl or TCE. (Note when the N-cap is methyl succinyl the C- 30 terminus R group cannot be Methyl.) Although DMF is given as the solvent, other solvents such as DMSO, CH₃CN, or NMP (or mixtures thereof) may be substituted therefor. Pyridine, Et₃N or other bases may be substituted for piperidine in deprotecting the growing peptide chain protected amino terminus. Similarly, although HBTU is given in the diagram above as the activating agent, other activating agents

such as DCC, DIC, DCC + HOBr, OSu, activated esters, azide, or triphenyl phosphoryl azide may be used. Additionally, the protected peptide acid chloride or acid bromide may be used to couple directly to the amino acid or peptide fragment. On completion of the Oligopeptide assembly the N-terminus deprotected and the C-5 terminus protected peptide is ready to accept the desired N-cap.

General Procedure for the Preparation of N-cap Oligopeptide via Solution Phase Synthesis

When constructing the N-capped Oligopeptide by solution phase synthesis, the 10 N-cap needs to be synthesized by a slightly modified procedure (Fig. 4). First the C-terminus of the Fmoc oligopeptide needs to be protected with an acid labile or hydrogenation sensitive protecting group compatible with the selective deprotection of the C-terminus over the N-cap. Then the Fmoc protecting group needs to be removed from the oligopeptide to reveal the N-terminus. With the N-terminus 15 deprotected, and the C-terminus protected the Oligopeptide is reacted with the activated hemiester of the desired N-cap. The N-cap can be activated using methods for activating amino acids such as DCC or HATU in base and an appropriate solvent. Alternatively, where the methyl-hemisuccinate is used, the coupling may also be done via methyl hemisuccinyl chloride (or other acid halide) (Fig. 4) using an inert solvent 20 in the presence of an organic or inorganic base, such as DIEA, triethylamine or Cs₂CO₃. One example of such a synthesis can be by reacting methyl-hemisuccinate and Oligopeptide 38 benzyl ester. The coupling method can be any one of the methods generally used in the art (see for example: Bodanszky, M., *The Practice of Peptide Synthesis*, Springer Verlag, 185 (1984); Bodanszky, M., *Principles of Peptide Synthesis*, Springer Verlag, 159 (1984). The benzyl group than can be removed by 25 catalytic hydrogenation providing the desired N-cap methyl-succinyl form of Oligopeptide 38. Other examples but not limited to of suitable, selectively removable C-terminal protecting groups can be tBu, alkoxy-methyl and TCE. Other methods of accomplishing this step are described in the literature.

30 Any combination of the above method can be considered, such as "fragment condensation" of di-, or tripeptides. The reaction conditions are well known in the art and detailed in the citations given. The advantage of the above described methods is the facile purification of the product produced by solution phase synthesis.

PRODRUG CONJUGATE

General Methods for the Conjugation and Deprotection steps

The N-cap form of Oligopeptide-therapeutic agent (prodrug conjugates) described in this invention can be synthesized by coupling Fmoc form (means Fmoc is attached to the N-terminus of the Oligopeptide) of the oligopeptide with daunorubicin or any appropriate therapeutic agent using any of the standard activating reagents used in peptide synthesis (Fig. 6). The solvent may be Toluene, ethyl acetate, DMF, DMSO, CH₃CN, NMP, THF, DCM or any other suitable inert solvent as is known in the art and the reagents are soluble therein. The preferred solvents are DMF and NMP. The appropriate temperature range is -25 to +25°C, with ambient temperature being preferred. The activating agent may be selected from one of the following: PyBOP, HBTU, HATU, EDC, DIC, DCC, DCC+HOBT, OSu activated esters, azide, or triphenylphosphorylazide. HBTU or HATU is the preferred activating agent. Alternatively, the acid chloride or the acid bromide of the protected peptide can also be used for this coupling reaction. 2-4 equivalent, advantageously 2-2.5 equivalent of a base is required for the coupling reaction. The base can be selected from inorganic bases such as CsCO₃, Na- or K₂CO₃, or organic bases, such as TEA, DIEA, DBU, DBN, DBO, pyridine, substituted pyridines, N-methyl-morpholine etc., preferably TEA, or DIEA. The reaction can be carried out at temperatures between -15 °C to 50 °C, advantageously between -10 °C and 10 °C. The reaction time is between 5-90 minutes is advantageously 20-40 minutes. The product is isolated by pouring the reaction mixture into water and filtration of the precipitate formed. The crude product can be further purified by recrystallization from DCM, THF, ethyl acetate, or acetonitrile, preferably from dichloromethane or acetonitrile. The isolated Fmoc form of Oligopeptide therapeutic agent conjugate is then deprotected over 2-90 minutes preferably 3-8 minutes using a ten to hundred fold excess of base at temperature between -10 °C and 50 °C. Ideally 5-60 equivalents of the base are preferred. Piperidine is the preferred base to deprotect Fmoc groups. The deprotected amino terminus of the Oligopeptide therapeutic agent conjugate is acylated by a diacid anhydride as an activated hemi-ester to give the final N-cap form of Oligopeptide-therapeutic agent (prodrug).

Alternatively, the final prodrug can be similarly prepared from the protected N-cap form of the Oligopeptide such as a methyl-hemi ester form of succinyl-N-cap

Oligopeptide and conjugated to a therapeutic agent. This method is illustrated in Fig. 6.

The protected N-Cap-oligopeptide therapeutic agent is now deprotected by methods compatible to the stability of the therapeutic agent. For example, for 5 antracyclines we protect with a methyl group and deprotect with an esterase. For others therapeutic agents we might select benzyl protecting groups and catalytic hydrogenation to deprotect.

The salt form of the negatively charged N-cap Oligopeptide therapeutic agent is carried out with a solvent selected from the following group: alcohol (including 10 methanol, ethanol, or isopropanol), water, acetonitrile, tetrahydrofuran, diglyme or other polar solvents. The sodium source is one molar equivalent of NaHCO₃, NaOH, Na₂CO₃, NaOAc, NaOCH₃ (in general sodium alkoxide), or NaH. An ion exchange column charged with Na⁺ (such as strong or weak ion exchangers) is also useful for this last step of making the salt form of the N-cap Oligopeptide therapeutic agent 15 when appropriate. Sodium is described in this application as an example only. Any pharmaceutically acceptable salt can be used for negatively charged N-caps.

Generally, the prodrug may be converted to a pharmaceutically acceptable salt form to improve solubility of the prodrug. The N-cap-oligopeptide therapeutic agent is neutralized with a pharmaceutically acceptable salt e.g., NaHCO₃, Na₂CO₃, NaOH 20 tris(hydroxymethyl)aminomethane, KHCO₃, K₂CO₃, CaCO₃, NH₄OH, CH₃NH₂, (CH₃)₂NH, (CH₃)₃N, acetyltriethylammonium, preferred salt form of prodrug is sodium. Preferred neutralizing salt NaHCO₃.

It is well documented, anthracycline type molecules, including doxorubicin and daunorubicin form gels in organic solvents in very low concentrations (Matzanke, 25 B. F., et al., Eur. J. Biochem., 207:747-55 (1992); Chaires, J. B., et al., Biochemistry, 21:3927-32 (1982); Hayakawa, E., et al., Chem. Pharm. Bull., 39:1282-6 (1991). We have found this to be a considerable obstacle to getting high yields of clean product when making peptide anthracycline conjugates. The gel formation contributes to the formation of undesirable side reactions. One way to minimize this problem is to use 30 very diluted solutions (1-2%) for the coupling reaction, however it is not practical in a process environment (large amounts of waste, complicated isolation). To overcome this problem we have invented a method wherein urea and other chaotropic agents are used to break up the strong hydrophobic and hydrogen bonding forces forming the gel. Thus if the coupling reaction is carried out in a urea containing solvent,

advantageously 20% to saturated solution of urea in DMF or NMP, the side reactions can be kept below 2% even if the concentration of reactants exceeds 10%. This invention makes practical the conjugation step at high concentrations and produces good yields and improved purity over the procedures that do not use urea as other 5 isotopic agents.

General Enzyme Method

Hydrolyses of protected N-cap-oligopeptide therapeutic agents to the full N-cap compound catalyzed by acids or bases leads to complex reaction mixture due to 10 the lability of many therapeutic agents even under moderately acidic or basic conditions. We have found that enzymes can promote the hydrolysis without destroying the substrate or the product. Enzymes suitable for this reaction can be selected from esterases, lipases and can be in their natural, water soluble forms or immobilized by cross coupling, or attachment to commercially available solid 15 supporting materials. Of the soluble enzymes evaluated *Candida Antarctica "B"* lipase (Altus Biologics) is especially useful. Examples of enzymes immobilized by cross coupling is ChiroCLEC-PCTM (Altus Biologics). *Candida Antarctica "B"* lipase (Altus Biologics) can be immobilized by reaction with NHS activated SepharoseTM 4 Fast Flow (American Pharmacia Biotech). The pH of the reaction mixture during the 20 hydrolysis is carefully controlled and maintained by a pH-stat between 5.5 and 7.5, advantageously between 5.7 and 6.5, via controlled addition of NaHCO₃ solution. When the reaction is completed the product is isolated by lyophilization of the filtered reaction mixture. The immobilized enzymes remains on the filter cake and can be reused if desired.

25

General Allyl Ester Method

The prodrug can also be prepared via coupling allyl-hemiesters form of the N-cap oligopeptide with a therapeutic agent and then liberating the free acid from the 30 conjugate. Fig. 8 illustrates this process with Succinyl-β-Ala-Leu-Ala-Leu and doxorubicin.

The coupling of allyl-succinyl-β-Ala-Leu-Ala-Leu with doxorubicin can be carried out via any one of the oligopeptide conjugation methods.

Allyl-succinyl- β -Ala-Leu-Ala-Leu-doxorubicin can also be synthesized by reacting allyl hemisuccinate, which was prepared via known methods (Casimir. J. R., et.al., *Tet. Lett.* 36/19 3409 (1995)), with β -Ala-Leu-Ala-Leu-doxorubicin similarly as coupling of the protected tetrapeptide precursors to doxorubicin was described in the previous methods, shown in Fig. 4. Suitable inert solvents are THF, dichloromethane, ethyl acetate, toluene, preferably THF from which the acid form of the product precipitates as the reaction progresses. The isolated acid is converted to its sodium salt as described earlier. Reaction times vary between 10-180 minutes, advantageously 10-60 minutes, at temperatures between 0-60 °C, preferably 15-30 °C.

10 Removal of the allyl-group can be done with Pd (0), or Ni(0), advantageously Pd(0) promoted transfer of the allyl group to acceptor molecules, as it is well known in the art and documented in the professional literature (Genet, J-P, et al., *Tet. Lett.*, 50, 497, 1994; Bricout, H., et.al. *Tet. Lett.*, 54:1073 (1998), Genet, J-P. et.al. *Synlett*, 680 (1993); Waldmann, H., et.al., *Bioorg. Med. Chem.*, 7:749 (1998); Shaphiro, G., Buechler, D., *Tet. Lett.*, 35:5421 (1994)). The amount of catalyst can be 0.5-25 % mol to the substrate.

General Trityl or Substituted Trityl Method

20 The prodrug may also be synthesized via the method shown in Fig. 7 this approach utilizes an R'-tetrapeptide, where R' is trityl or substituted trityl. The Coupling of R'-tetrapeptide with a therapeutic agent can be carried out via any one of the methods described earlier for conjugation of an protected oligopeptide with a therapeutic agent at 30-120 minutes at 0-20°C.

25 Removal of trityl or substituted trityl group can be achieved under acidic conditions to give the positively charged prodrug. This positively charged prodrug is N-capped as illustrated in Fig. 4 and described earlier. The trityl deprotection can be accomplished with acetic acid, formic acid and dilute hydrochloric acid.

30 The prodrug can be converted into succinyl or glutaryl oligopeptide 38 therapeutic agent by reacting with succinic anhydride. Succinyl or glutaryl Oligopeptide 38 therapeutic agent can be converted to any pharmaceutically acceptable salt. The solvent for coupling step DMF, DMSO, CH₃CN, NMP, or any other suitable solvent is known in the art.

General Inverse Direction Solid Phase Conjugation Method

The prodrug compound of the present invention can be synthesized by using solid phase chemistry via "step wise" inverse (from the N-terminal to the C-terminal) direction methods.

5 One way is to use resins to immobilize a succinyl-hemi ester, for example succinyl-mono-benzyl ester or -allyl ester. Examples of resins could be selected are "Wang Resins" (Wang, S. S., J. Am. Chem. Soc., 95:1328 (1973); Zhang, C., Mjaili, A. M. M., Tet. Lett., 37:5457(1996)), "Rink Resins" (Rink, H., Tet. Lett., 28:3787 (1987)), "Trityl-, or substituted-trityl Resins" (Chen, C., et.al., J. Am. Chem. Soc., 10 116:2661 (1994); Bartos, K. et.al., Peptides, Proc. 22nd European Peptide Symposium (1992); Schneider, C. H.; Eberle, A. N. (Eds.), ESCOM, Leiden, pp. 281 (1993). The immobilized ester is then deprotected and reacted with similarly C-terminal protected β -alanine. These steps are then repeated with leucine-, alanine, and finally leucine esters, followed by the coupling of doxorubicin to the immobilized succinyl-
15 tetrapptide. The molecule is then liberated from the resin by using mildly acidic conditions to form free succ-Oligopeptide 38-Doxorubicin. This methodology is represented on the scheme of Fig. 9. Another version of phase synthesis would be if the succinyl tetrapeptide ester is immobilized. It is then C-terminally deprotected, followed by the coupling step to doxorubicin and finally liberated from the resin as
20 represented on the scheme of Fig. 9. The acid form of the prodrug molecule is converted finally into its sodium salt as described above.

Specific Compounds

25 Compounds of the invention include the prodrugs, Suc- β Ala-Leu-Ala-Leu-Dox, Suc- β Ala-Leu-Ala-Leu-Dnr, and Glutaryl- β Ala-Leu-Ala-Leu-Dox.

Additionally, the following intermediate compounds, important to the process of preparation of the prodrugs of the invention, are claimed.

30 **Intermediates:**

β Ala -Leu-Ala-Leu-Dox

Trityl- β Ala -Leu-Ala-Leu-Dox

Diphenylmethyl- β Ala -Leu-Ala-Leu-Dox

Benzylloxycarbonyl- β Ala -Leu-Ala-Leu-Dox
Fmoc- β Ala -Leu-Ala-Leu-OBn
 β Ala -Leu-Ala-Leu-OBn
Methyl-succinyl- β Ala -Leu-Ala-Leu-OBn
5 Methyl-succinyl- β Ala -Leu-Ala-Leu
Fmoc- β Ala -Leu-Ala-Leu
Fmoc-Thi-Tyr-Gly-Leu
Fmoc- β Ala -Leu-Ala-Leu-Dnr
Fmoc-Thi-Tyr-Gly-Leu-Dnr
10 Suc-Thi-Tyr-Gly-Leu-Dnr
Gl- β Ala -Leu-Ala-Leu-Dox
 β Ala -Leu-Ala-Leu-Dox Lactate
Allyl-succinyl- β Ala -Leu-Ala-Leu-Dox
Suc- β Ala -Leu-Ala-Leu
15 Methyl esters of Suc- β Ala -Leu-Ala-Leu
Fmoc- β Ala -Leu-Ala-Leu-Dox
Methyl-succinyl- β Ala -Leu-Ala-Leu-Dox, and
Allyl-hemi succinate.

20

EXAMPLES**Example 1:****Screening of potential prodrugs with truase and human blood**

Based on HPLC analysis of digestion products, activation of peptidyl-toxin to free toxin occurs via a series of enzyme catalyzed cleavage reactions. For example, 25 the N-capped tetrapeptidyl toxin, succinyl- β Alanyl-leucyl-alanyl-leucyl-doxorubicin is converted to leucyl-doxorubicin in extracts of carcinoma cells or carcinoma cell conditioned media in two steps catalyzed by at least two enzymes. Initial endopeptidase cleavage occurs between the AA³ (P1) and AA² (P1') amino acids to 30 yield alanyl-leucyl-doxorubicin. Subsequently, exopeptidase removes alanine to give leucyl-doxorubicin which is known to be taken up into cells where the active toxin, doxorubicin, is released.

A good candidate for a higher therapeutic index N-capped peptidyl-toxin prodrug should be activated by cancer cells but be relatively stable in whole human blood. Three different preparations of carcinoma were used to screen various N-capped peptidyl-toxins. These three preparations were as follows:

- 5 (a) MCF 7/6 (breast carcinoma) cell homogenate
- (b) MCF 7/6 (breast carcinoma) conditioned media, and
- (c) HeLa (cervical carcinoma) cell extract anion exchange fraction pool.

Compounds which could be hydrolyzed to a single amino acid toxin conjugate were further tested for stability in whole human blood.

- 10 Test samples were incubated at 37°C for 2 hr with the three different preparations of carcinoma enzyme and with whole blood, extracted with acetonitrile, and analyzed by HPLC using fluorescence detection. With few exceptions, results for carcinoma enzyme cleavage were the same for a partially purified fraction from HeLa cells, MFC 7/6 cell homogenate, or MCF 7/6 conditioned media.

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Preparation of carcinoma cell enzyme solutions:

- (a) MCF 7/6 cell homogenate:

MCF 7/6 cells were grown to confluence in a serum free medium containing DMEM:F12 (1:1), 50 mg/L bovine serum albumin, ITS-X, and Lipid Concentrate.

- 20 100 mL of cells were harvested by centrifugation at 4°C 10,000xg, for 20 min and decanting the supernatant. The pellet was resuspended in 2 mL phosphate buffered saline (*Gibco*) and centrifuged at 18,000xg for 10 min. After decanting the supernatant, the cells (approximately 300 µL wet) were homogenized by grinding in 1.7 mL 10 mM pH 7.2 HEPES buffer (sodium salt). The homogenate was
- 25 centrifuged at 18,000xg at 4°C for 5 min and the supernatant was aliquoted and stored at ≤-20°C for subsequent use in the compound screen.

- (b) MCF 7/6 conditioned media:

- 30 MCF 7/6 cells were grown to confluence in DMEM/F12 (1:1) medium containing 10 % fetal bovine serum, 0.05% (w/v) L-glutamine, 250 IU/mL penicillin, and 100 µg/mL streptomycin. Cells were then washed twice with phosphate buffered saline and incubated 24 hr at 5% CO₂, 37°C, in DMEM/F12 (1:1), 0.02% BSA, ITS-X. The conditioned media was then decanted and, using a stirred cell apparatus with

a YM10 (10,000 MW cutoff) ultrafiltration membrane (*Millipore*), exchanged once with 10 mM HEPES buffer, pH 7.2 and concentrated twenty-fold. This solution was stored in aliquots at -20°C for use in the compound screen.

5 (c) HeLa Cell anion exchange fraction pool:

Thirty billion commercially produced HeLa Cells (human cervical carcinoma, *Computer Cell Culture Center*, Seneffe, Belgium) were homogenized with a sonicator and with a Dounce homogenizer in 108 mL of aqueous lysis solution. The lysis solution contained 0.02% w/v Triton X-100, 0.04% w/v sodium azide, and a cocktail 10 of protease inhibitors (2 tablets/ 50 mL Complete™, EDTA-free tablets, *Roche Molecular Biochemicals*). The cell homogenate was centrifuged 30 minutes at 4°C at 5000xg and the pellet was homogenized in a second 108 mL of lysis solution using a Dounce homogenizer and centrifuged as before. The supernatants were combined and centrifuged for 90 min at 60,000xg at 4°C.

15

Chromatography

A portion of the ultracentrifugation supernatant was diluted 2-fold with a 20 mM triethanolamine-HCl pH 7.2 buffer containing 0.01% (w/v) Triton X-100 and 0.02% (w/v) sodium azide (equilibration buffer). Thirty mL of the resulting solution, 20 corresponding to approximately 180 mg of protein, was loaded at 4°C on a 2.6 x 9.4 cm Source™15Q (*Amersham Pharmacia Biotech*) low pressure anion exchange chromatography column (1 mL/minute). The column was then washed with 250 mL of the equilibration buffer and a flow rate of 1 mL/minute. Proteins were eluted in a NaCl linear concentration gradient (0-0.5 M in the equilibration buffer, total volume 25 of the gradient was 1000 mL) at a flow rate of 3 mL/minute. Two-minute fractions were collected and used for enzyme activity determination using β -alanyl-leucyl-alanyl-leucyl-doxorubicin as the substrate. Its transformation into L-alanyl-L-leucyl-doxorubicin was quantified by reverse phase high performance liquid chromatography utilizing fluorescence detection of the anthracycline moiety. The fractions containing 30 the highest activity levels were pooled (fractions #43-46; ~0.13 M NaCl), supplemented with protease inhibitors (Complete™, EDTA-free tablets, *Roche Molecular Biochemicals*), and stored as aliquots at -80°C.

Whole Human blood

Human blood was collected using commercial acid buffered citrate whole blood collection tubes.

5 Compound screen

Test compounds were incubated for 2 hr at 37°C at a concentration of 12.5 µg/mL with the following enzyme solutions:

- a) MFC 7/6 cell homogenate diluted 1:27 in 10 mM HEPES, 1 mM CoCl₂, pH 7.2
- 10 b) MFC 7/6 conditioned media
- c) Hela Cell anion exchange fraction pool 1 diluted 1:57 in 10 mM HEPES, 1 mM CoCl₂, pH 7.2.
- d) Whole human blood containing 1 mM CoCl₂

15 Following incubation, three volumes of acetonitrile were added to stop the reaction and remove protein from the mixture. The sample was centrifuged at 18,000g for 5 minutes and 100 µL of supernatant was mixed with 300 µL of water prior to analysis by HPLC.

20 For HPLC analysis 50 µL of sample was injected on a 4.6 x 50 mM 2µ TSK Super-ODS chromatography column at 40°C and eluted with a 3 minute linear gradient from 26% to 68% acetonitrile in aqueous 20 mM ammonium acetate pH 4.5 buffer at 2 mL/min. Detection was by fluorescence using an excitation wavelength of 235 nM and an emission wavelength of 560 nM.

25 Oligopeptides that were cleaved by the trouse under the given conditions and were stable in human blood are shown in Figs. 9A-9C.

Example 2:**Specificity for trouse is provided by a non-genetically encoded amino acid at position P2**

30 Specificity is afforded by incorporation of a non-genetically encoded rather than a genetically encoded amino acid at position P2. Specifically, (succinyl N-capped)-(Oligopeptide 38)-daunorubicin, which contains the non-genetically encoded amino acid β-alanine at position P2, was incubated for 2 hr at 37°C with each enzyme

preparations prepared as described in Example 1. The extent of cleavage was then estimated by HPLC analysis of the resulting mixtures. These results were compared to results for the same incubations performed with the same compound except for a substitution of the genetically encoded amino acid L-alanine position at P2. The 5 extent (rate) of cleavage by cell homogenate was 1.3 fold greater for the P2 L-alanine compound versus the P2 β -alanine compound. The extent of cleavage by conditioned medium was about the same for the two compounds. However, with the partially purified trypsin preparation, the extent of cleavage of the P2 L-alanine compound was only 0.6 fold that of the P2 β -alanine compound. These results suggest that the 10 presence of L-alanine at P2 may have provided a second cleavage site for the cruder mixtures of enzymes; thus reducing the likelihood that, *in vivo*, release of the active drug would be localized to tumor tissue.

Example 3:**15 The Prodrug is Effective and Well-Tolerated in Tumor Xenograft Models**

(Succinyl N-Cap)-(Oligopeptide 38)-Dox therapeutic agent has proven to be efficacious in inhibiting the growth of human tumors in several nude mouse xenograft models, including the estrogen-dependent MCF-7/6 mammary tumor and the adriamycin-resistant colorectal carcinomas CXF280/10 and LS-174T. For example, 20 when groups of 10 mice with subcutaneously-implanted LS174T tumors were treated with five weekly intravenous doses of (Succinyl N-Cap)-(Oligopeptide 38)-Dox therapeutic agent, a significant, dose-dependent, replicable extension in the Mean Day of Survival (MDS) was observed, as well as in decreased size of the tumor (tumor volume) compared with vehicle-treated controls (Group 1) at doses of 57 (Group 2), 25 64 (Group 3) and 71 (Group 4) mg/kg of (Succinyl N-Cap)-(Oligopeptide 38)-Dox therapeutic agent, with the highest dose being equivalent to 40 mg/kg of doxorubicin (See Fig. 11). The drug was safe and well-tolerated under repeat-dose levels and frequencies of dosing that demonstrated anti-tumor efficacy. Some dose-dependent body weight loss was observed. In supporting studies, kidney toxicity and 30 myelosuppression were not observed at doses of up to 106.8 mg/kg of (Succinyl N-Cap)-(Oligopeptide 38)-Dox therapeutic agent.

Example 4**The Prodrug is Safer and More Effective than Comparators**

Significantly higher doses of (Succinyl N-Cap)-(Oligopeptide 38)-Dox therapeutic agent could be administered compared with doxorubicin, achieving efficacy without significant toxicity in the LS-174T human colorectal carcinoma xenograft model. (Succinyl (Group 5) N-Cap)-(Oligopeptide 38)-Dox therapeutic agent, at efficacious doses of 49 (Group 3), 57 (Group 4) and 64 mg/kg (Group 5); showed superior efficacy compared to doxorubicin at 3.0 mg/kg (Group 2) and saline (Group 1), in inhibiting the rapidly-growing adriamycin-resistant LS-174T tumor (Fig. 12). Dose limiting toxicity (cardiotoxicity and myelosuppression) has been observed with repeated administration of doxorubicin at or above 3 mg/kg (Group 2). Thus we have demonstrated that higher doses of (Succinyl N-Cap)-(oligopeptide 38)-Dox therapeutic agent than doxorubicin can be administered, favoring tumor inhibition over systemic toxicity.

15 **Example 5**

β AlaLeuAlaLeu-Dox Aggregation

Poorly soluble anthracycline drugs have been shown to form aggregates when prepared in aqueous buffers. Menozzi, et al., *Self-association of doxorubicin and related compounds in aqueous solutions*, *J. Pharmaceut. Sci.*, 73(6):766-770 (1984).

20 Confalonieri, C. et al., *The use of new laser particle sizer and shape analyser to detect and evaluate gelatinous microparticles suspended in reconstituted anthracycline infusion solutions*, *J. Pharmaceut. Biomed. Anal.*, 9(1):1-8 (1991). An estimation of β AlaLeuAlaLeu-Dox aggregate size in a 17.4 μ Mol/ml aqueous solution by attempting to filter these solutions through Amicon CentriconTM filter units.

25 Doxorubicin (17.4 μ Mol /ml) and β AlaLeuAlaLeu-Dox (17.4 μ Mol /ml) were each dissolved in distilled water and placed into Centricon filters with 3,000, 10,000, 30,000 and 50,000 molecular weight cutoff (MWCO). Each filter unit was centrifuged for 2 hr at 1500g force. The amount of the drug retained and passing through the filter was quantitated at λ 475 nm and converted to a percent. Table 1

30 below shows that 81 % of the doxorubicin passed through the 3,000 MWCO filter while only 5 % of the conjugate, β AlaLeuAlaLeu-Dox passed through the 3,000 MWCO filter. The data also show that the 50,000 MWCO unit retains over 40 % of the β AlaLeuAlaLeu-Dox. These data demonstrate that a significant percentage of β AlaLeuAlaLeu-Dox aggregates were larger than 50 kD (>50 molecules/aggregate).

This demonstrates that at the specified dose of Example 6, below, the conjugate was in an aggregated state. This data, therefore, support the hypothesis that Oligopeptide 38-Doxorubicin Therapeutic Agent aggregates contribute to the acute toxicity seen in this positively charged molecular aggregates.

5

Table 1

	3000 MWCO		10000 MWCO		30000 MWCO		50000 MWCO	
	Filt.	Ret.	Filt.	Ret.	Filt.	Ret.	Filt.	Ret.
Dox	81%	10%	82%	2%	n.d.	n.d.	93%	0.5%
Conj.	4.9%	89%	10%	76%	36%	64%	53%	43%

Dox: doxorubicin; Conj: N- β -Ala-L-Leu-L-Ala-L-Leu-Doxorubicin;

Example 6

10 Intravenous Injection of β AlaLeuAlaLeu-Dox in Mice

It is known that acute toxicity likely occurs through the interaction of positively charged polymers, such as protamines, polylysine, or their aggregates, and the luminal surface of blood vessels. DeLucia III, A., et al., *Efficacy and toxicity of differently charged polycationic protamine-like peptides for heparin anticoagulation reversal*, *J. Vasc. Surg.*, 18:49-60 (1993). Ekrami, H. M. and Shen, W. C., *Carbamylation decreases the cytotoxicity but not the drug-carrier properties of polylysines*, *J. Drug Targ.*, 2:469-475 (1995). It has been further shown that heparin reduces the toxic effects of protamine sulfate on rabbit myocardium. Wakefield, T. W., et al., *Heparin-mediated reductions of the toxic effects of protamine sulfate on rabbit myocardium*, *J. Vasc. Surg.*, 16:47-53 (1992). To test the hypothesis that the acute toxicity seen here was due to positively charged prodrug aggregates, β AlaLeuAlaLeu-Dox (174 μ Mol /ml) was given to mice following a 1 hr pretreatment with either 4,000 I.U. heparin iv as compared to control (iv). Table 2 shows that following heparin, a formerly acutely lethal dose of β AlaLeuAlaLeu-Dox was significantly less toxic.

These data support the hypothesis that the acute toxicity is due to a positively charged aggregate causing a similar effect to that seen for protamines or polylysine. Negatively and neutrally charged prodrugs of the invention overcome this undesirable side effect.

5

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Table 2

ROUTE OF Pretreatment	HEPARIN DOSE LEVEL (I. U.)	Survival time (days) [Proportion]	Acute toxicity (Proportion)
<i>Control (iv)</i>	0	0	5/5
<i>i.p.</i>	4000	>9	3/8
		[5/8]	
	8000	>11 [3/3]	0/3
<i>i.v.</i>	4000	>11 [2/3]	1/3

In agreement with the aforementioned hypothesis, capping the terminal amino group of β AlaLeuAlaLeu-Dox with a negatively charged moiety resulted in the complete disappearance of the acute toxicity effect at dose levels as high as 250 mg doxorubicin, HCl eq./Kg.

As evidence of this, in a related experiment, all animals survived up to 8 days when three to five mice per group were treated with an iv bolus of 250 mg/kg (Dox-HCl e.g.) succinyl β AlaLeuAlaLeu-Dox and glutaryl β AlaLeuAlaLeu-Dox.

20

Analytical Methods for the remaining examples

The peptide sequences, synthesized using either solid or solution phase approaches, were used without further purification if the analytical HPLC (methods A, B & D) showed the crude product to be greater than 80% pure. If not, the material was purified using preparative HPLC Method C.

30 HPLC Meth d A

Analytical HPLC analyses were performed on Waters 2690 using a C-18 column (4 μ m, 3.9 x 150mm ID, flow rate 1mL/min) eluting with a gradient of solvent A (0.1% TFA/H₂O) and solvent B (0.1% TFA/ACN) and the data was processed at λ 254 nm using the Waters Millennium system. Analytical HPLC gradient started with 5 90% of solvent A and ended with 100 % of solvent B over a period of 14 minutes (linear). Purity of the compounds for this method and the following ones was assessed as the relative percentage area under the curve of the peaks.

HPLC Method B

10 Analytical HPLC analyses were performed on Waters 2690 using a C-8 column (3.5 μ m, 4.6 x 150mm ID, flow rate 1mL/min) eluting with a gradient of solvent A (80% 20mM ammonium formate and 20% acetonitrile) and solvent B (20% 20mM ammonium formate and 80% acetonitrile) and the data was processed at λ 254 nm using the Waters Millennium system. Analytical HPLC gradient started with 15 100% of solvent A to 100% of solvent B over a period of 30 minutes (linear).

HPLC Method C

Preparative purification of crude products was achieved using Waters Delta Prep 4000 system using a C-4 column (15 μ m, 40 x 100mm ID, flow rate 30 mL/min) 20 eluting with a gradient of solvent A (H₂O), and solvent B (MeOH). The preparatory HPLC gradient started with 80% of solvent A and goes to 100 % of solvent B over a period of 70 minutes (linear). The data was processed at λ 254 nm using the Waters Millennium System.

25 **HPLC Method D**

Analytical HPLC was accomplished on a Hewlett Packard instrument:
Column: TSK superODS (TosoHaas); solvent A (TFA 0.1% in water) solvent B (TFA 0.1% in acetonitrile); gradient: 30 to 36% of B in 2 minutes, 36 to 41% of B in 10 minutes, 41 to 90% of B in 3 minutes, 5 minutes at 90% B, detection wavelength λ 30 254 nm.

NMR and MS

Additional structural determinations were done by NMR and MS techniques and the results supported the claimed compounds.

TLC Method

5 TLC analysis was carried out on silica gel 60F-254nm-0.25mm plates (Merck) with DCM/MeOH/H₂O/Formic acid 88% 85/15/1/2 for elution.

Ninhydrin Test

10 Solution A: ninhydrin (500 mg) in ethanol (10 mL); Solution B: phenol (80 mg) in ethanol (20 mL); Solution C: KSCN (0.01M in H₂O, 2 mL) in pyridine (100 mL). Method: A few milligrams of product were introduced in a test tube, and two drops of Solution A, two drops of Solution B, then two drops of Solution C were added. The mixture was left in a boiling water bath for five minutes. In the presence of a free amine the solution becomes purple.

15

Specific Oligopeptide Synthetic Examples

Sources of commercially available reagents

20 Doxorubicin and Daunorubicin were supplied by Meiji (Japan), Pd(PPh₃)₄ by Strem chem (Newburyport, MA), PEG by Shearwater(Huntsville, Alabama), solvents, HATU by Aldrich (Milwaukee, WI); all resins and amino acids were either supplied by ABI (Foster City, CA), Novabiochem (San Diego, CA), Advanced ChemTech (Louisville, KY), Peptide International (Louisville, KY), SynPep (Dublin, CA).

25 **Example 7**

Fmoc form of Oligopeptide 38 benzyl ester [Fmoc- β -Ala-Leu-Ala-Leu-OBn]

30 The Fmoc form of Oligopeptide 38 (24.34 g, 0.04 mol) was added into a round bottom flask with DMF (350 mL) and a magnetic stirrer. After the tetrapeptide was dissolved, benzyl bromide (4.76 mL, 0.04 mol), followed by cesium carbonate (13.04 g, 0.04 mol), was added to the solution with stirring. The reaction mixture was stirred at room temperature for 1.5 hrs. Then, the reaction mixture was slowly poured into a flask with 450 mL of iced water. A large amount of white solid precipitated out which was collected by suction filtration. The product was washed with water (2x200 mL)

and placed in a vacuum desiccator. The product (24.2 g, 87%) was identified by HPLC (Purity: 95%). MS m/z calcd. for $C_{40}H_{50}N_4O_7$ 698.4, found 699.5.

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Example 8**Oligopeptide 38 benzyl ester [β -Ala-Leu-Ala-Leu-OBn]**

In a round bottom flask (25 mL), Fmoc form of Oligopeptide 38 benzyl ester (0.7 g, 1.0 mmol) was dissolved in 5 mL of anhydrous DMF. Piperidine (1.2 mL, 12.1 mmol) was added to the solution and the mixture was stirred at room temperature for 25 minutes. The reaction was quenched with water (6 mL) and extracted with ethyl acetate (2x10 mL). The combined organic layer was further washed by water (2x5 mL), brine (5 mL) and dried over sodium sulfate. A white solid (0.8 g) was obtained after removal of solvent. The purity of the product was only 67%. MS m/z calcd. for $C_{25}H_{40}N_4O_5$ 476.3, found 477.2.

Example 9**Methyl Succinyl-N-cap form of Oligopeptide 38 benzyl ester [mono-Methyl-succinyl- β -Ala-Leu-Ala-Leu-OBn]**

In a round bottom flask (250 mL), methyl hemisuccinate (3.19 g, 24.2 mmol) was dissolved in anhydrous DMF (50 mL). DIEA (4.22 mL, 24.2 mmol) followed by HBTU (9.17 g, 24.2 mmol) were added into the solution. The mixture was stirred at room temperature for 45 minutes. To this mixture was added a solution of Oligopeptide 38 benzyl ester (crude, containing 10.14 g, 21.3 mmol) in anhydrous DMF (150 mL). The mixture was continually stirred at room temperature for 2.5 hrs. Then, the reaction mixture was slowly poured into a flask with 200 mL of iced water while stirring. A large amount of white solid precipitated out which was extracted by ethyl acetate (3x200 mL). The combined organic layer was further washed by water (2x200 mL), brine (200 mL) and dried over sodium sulfate. A white solid was obtained after removal of solvent. Recrystallization of this crude product in ethyl acetate afforded 7.53 g of product (60%) with purity of 80%. MS m/z calcd. for $C_{30}H_{46}N_4O_8$ 591.4, found 590.33.

Example 10

Methyl Succinyl-N-cap form of Oligopeptide 38 [Methyl succinyl- β -Ala-Leu-Ala-Leu]

Methyl succinyl-N-cap form of Oligopeptide 38 benzyl ester (1.0 g, 86% purity; 1.46 mmol) was added into an Erlenmeyer flask with 100 mL of methanol.

5 The solution was cloudy after stirred for a few minutes. 50 mL of methanol was added, but the solution was still not clear. The solution was transferred into a hydrogenation reaction vessel. To this vessel, Pd-C (90 mg, 10% wet, 50% water; 0.042 mmol) was added. After hydrogenation for 2 hours at room temperature, the reaction was stopped and the catalyst was filtered. A white solid (0.77 g, 78%) was 10 yielded after removal of solvents. MS *m/z* calcd. for $C_{23}H_{40}N_4O_8$ 501.2, found 500.3.

Example 11**Synthesis of N-cap Allyl-Hemisuccinate**

This molecule was prepared according the procedure of Casimir, J. R., et.al.

15 *Tet. Lett.* 36(19):3409, (1995). 10.07g (0.1 mol) succinic anhydride and 5.808g (0.1 mol) allyl-alcohol are refluxed in 100mL toluene for 6 hours. The reaction mixture is concentrated under reduced pressure. 15.5g; 98%. The resulting material was pure 20 enough to use in subsequent reactions. The purity and identity of the semi-solid product was confirmed by 1H NMR and ^{13}C NMR, by LC/MS.

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Example 12**Synthesis of Allyl-Succinyl-oligopeptide 38-Dox.**

In a round bottom flask (50 ml) N-Cap-Allylhemisuccinyl form of oligopeptide 38 (1g, 1.9 mmol) and doxorubicin (1.1g, 1.9 mmol) were dissolved in 25 anhydrous DMF (50 ml). After the mixture was stirred for 5 minutes, DIEA (0.66 ml, 3.8 mmol) followed by HATU (0.76g, 1.9 mmol) was added into the solution the mixture was stirred at room temperature for 2 hours. DMF was removed by a rotary evaporator and the residue was taken up in 4.0 ml 1:1 DCM: MeOH. To this 30 Solutions, 100 ml of ether was slowly added while stirring. A red precipitate was formed and collected by suction filtration. The sold was washed with ether (2x2 ml) and dried in a vacuum desicator to give Allyl-Succinyl-oligopeptide 38-Dox therapeutic agent with 90% HPLC purity by Method B.

Example 13**Preparation of SSLD from allyl-succinyl- β -Ala-Leu-Ala-Leu-doxorubicin**

To a stirred solution of 0.1g (0.095 mmol) allyl-succinyl- β -Ala-Leu-Ala-Leu-doxorubicin in 2 mL THF, under nitrogen atmosphere 0.05 g (0.095 mmol) 5 tetrakis(triphenylphosphine) palladium is added as a solid. After 10 minutes the precipitate formed during the reaction is filtered off, washed with THF. Dry weight: 0.1g. The solids have been identified by HPLC, 1 HNMR, LC/MS to be succinyl- β -Ala-Leu-Ala-Leu-Dox.

10

Example 14**Synthesis Fmoc form of Oligopeptide 38 [Fmoc- β -Ala-Leu-Ala-Leu]**

Fmoc form of Oligopeptide 38 was synthesized using solid-phase approach with standard Fmoc chemistry. A typical synthesis used Wang's alkoxy resin (0.60 15 mmol/gm loading). Fmoc-protected amino acids were used for solid-phase peptide synthesis. For a scale of 1mM peptide on resin, 3 equivalent of amino acid was preactivated with HBTU as the activating agent for 5 minutes before being added to the resin together with 2 equivalent of DIEA. The coupling reaction was carried out for 2 h and then washed with DMF (25 mL x 3) and DCM (25 mL x 3). The coupling 20 reaction was repeated using 2 equivalent of amino acid using similar conditions. The reaction progress was monitored using ninhydrin test and if the ninhydrin test indicated incomplete reaction after 2 h then the coupling step was repeated for a third time. Deprotection was accomplished using 20% piperidine in DMF for 15-20 minutes. The coupling step was repeated with the next amino acid until the desired 25 peptide was assembled on resin. The final cleavage of peptide from the resin was accomplished by treating the resin with a solution of 95%TFA and 5% water. After stirring the reaction mixture for 2h at rt, the resin was filtered under reduced pressure and washed twice with TFA. Filtrates were combined and the peptide was precipitated by adding 400 mL of cold ether. The peptide was filtered under reduced 30 pressure and dried to yield Fmoc form of Oligopeptide 38 (94% HPLC purity by method A). Crude peptide was used for the next step without any further purification.

Example 15

Synthesis of Fmoc form of Oligopeptide 98 [Fmoc-Thi-Tyr-Gly-Leu]

Fmoc form of Oligopeptide 98 was synthesized using solid-phase approach with standard Fmoc chemistry and Wang's alkoxy resin (0.60 mmol/gm loading).

Fmoc-protected amino acids and Fmoc-Thi-OH were used for solid-phase peptide

5 synthesis. For a scale of 1mM peptide on resin, 3 equivalent of amino acid was preactivated with HBTU as the activating agent for 5 minutes before being added to the resin together with 2 equivalent of DIEA. The coupling reaction was carried out for 2 h and then washed with DMF (25 mL x 3) and DCM (25 mL x 3). The coupling reaction was repeated using 2 equivalent of amino acid using similar conditions. The
10 reaction progress was monitored using ninhydrin test and if the ninhydrin test indicated incomplete reaction after 2 h then the coupling step was repeated for a third time. Deprotection was accomplished using 20% piperidine in DMF for 15-20 minutes. The coupling step was repeated with the next amino acid until the desired peptide was assembled on resin. The final cleavage of peptide from the resin was
15 accomplished by treating the resin with a solution of 95%TFA and 5% water. After stirring the reaction mixture for 2h at rt, the resin was filtered under reduced pressure and washed twice with TFA. Filtrates were combined and adding 400 mL of cold ether precipitated the peptide. The peptide was filtered under reduced pressure and dried to yield Fmoc form of Oligopeptide 98 (88% HPLC purity by method A). Crude
20 Fmoc form of Oligopeptide 98 was used for the next step without any further purification.

Example 16**Synthesis of Fmoc form of Oligopeptide 38-Dnr Therapeutic Agent [Fmoc- β -Ala-**

25 **Leu-Ala-Leu-Dnr]**

Daunorubicin.HCl (185 mg, 0.329 mmol) and Fmoc form of Oligopeptide 38 (200 mg, 0.329mmol) were dissolved at room temperature in anhydrous DMF (15 mL). To this rapidly stirred solution, DIEA (0.115 mL, 0.658 mmol) was added in one portion and the reaction mixture was stirred for 15 minutes at room temperature. The
30 reaction mixture was cooled to 0 °C using ice bath and 138 mg (0.362 mmol) of HATU was added slowly over 10 minutes. The reaction mixture was stirred for another 90 minutes at room temperature. Ice cold water (200 mL) was added to the reaction mixture which resulted in the formation of a red precipitate. The precipitate

was collected over a coarse frit, washed with 3 x 50 mL water and 3 x 50 diethyl ether and dried under reduced pressure to yield Fmoc form of Oligopeptide 38-Dnr Therapeutic Agent (94% yield, 95% HPLC purity by method A). This product was used for the next step without any further purification.

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Example 17**Synthesis of Fmoc form of Oligopeptide 98-Daunorubicin Therapeutic Agent (Fmoc-Thi-Tyr-Gly-Leu-Dnr)**

Daunorubicin.HCl (90 mg, 0.16 mmol) and Fmoc form of Oligopeptide 98 (120 mg, 0.16 mmol) were dissolved at room temperature in anhydrous DMF (15 mL). To this rapidly stirred solution, DIEA (0.56 mL, 0.16 mmol) was added in one portion and the reaction mixture was stirred for 15 minutes at room temperature. The reaction mixture was cooled to 0 °C using ice bath and 61 mg (0.16 mmol) of HATU was added slowly over 10 minutes. The reaction mixture was stirred for another 90 minutes at room temperature. Ice cold water (150 mL) was added to the reaction mixture which resulted in the formation of a red precipitate. The precipitate was collected over a coarse frit, washed with 3 x 50 mL water and 3 x 50 diethyl ether and dried under reduced pressure to yield Fmoc form of Oligopeptide 98-Daunorubicin Therapeutic Agent (94% yield, 91% HPLC purity by method A). This product was used for the next step without any further purification.

Example 18**Preparation of Fmoc-β-Ala-Leu-Ala-Leu-doxorubicin**

3.0g (5.17 mmol) doxorubicin hydrochloride and 3.15 g (5.17 mmol) Fmoc-βAla-Leu-Ala-Leu was dissolved at room temperature in 230 mL dry DMF under nitrogen. To this rapidly stirred solution, 1.798 mL (10.34 mmol) DIEA was added in one portion and the reaction mixture stirred at room temperature for 15 min. The reaction mixture was cooled to ~ -2° C in an ice/brine bath and 2.56 g (6.73 mmol) HATU in 58 mL DMF was added dropwise over 12 minutes with rapid stirring. The reaction mixture was stirred another 30 minutes at -2° C then 0.285 mL (1.64 mmol) DIEA was added in one portion. 580 mL water at 0° C was immediately resulting in formation of a flocculent red precipitate. The precipitate collected over a coarse glass frit, washed with 3 X 50 mL water and 3 x 50 mL diethyl ether in water and air dried

16 hours to yield 5.21 g Fmoc- β -Ala-Leu-Ala-Leu-Dox, 89.7% physical yield, 90.23% HPLC purity by Method B.

Example 19

5 Preparation of succinyl- β -Ala-Leu-Ala-Leu-Dox from Fmoc- β -Ala-Leu-Ala-Leu-dox

To a solution of 5.0 g (4.41 mmol) Fmoc- β -Ala-Leu-Ala-Leu in 230 mL dry DMF under nitrogen at room temperature, 21.8 mL (220 mmol) piperidine was added in one portion resulting in a color change from red to purple. The reaction mixture 10 was stirred 5 minutes at room temperature then cooled to *ca.* -20°C in a dry ice/acetone bath. 22.5 g (0.225 mol) succinic anhydride was then added in one portion with the reaction temperature maintained below -5°C. After *ca.* 2 minutes stirring at -10°C to -5°C the color changed from purple to red/orange. The cooling bath was removed and the reaction mixture stirred for 10 minutes. The reaction 15 mixture volume was then reduced to *ca.* 100 mL by rotary evaporation and then diluted with 125 mL chloroform. To this solution, 1400 mL diethyl ether was quickly added resulting in formation of a red precipitate. This precipitate was isolated on a medium glass frit and triturated with 5 X 200 mL diethyl ether to yield material of 89.13% HPLC purity. The precipitate was washed again with 1 x 20 mL diethyl ether 20 and air dried to yield 3.62 g succinyl- β -Ala-Leu-Ala-Leu-Dox 81% physical yield, 88.2% HPLC purity). This material was stirred in 30 mL water at 0°C and 33.98 mL (0.95 eq.) 0.1 M aq. NaHCO₃ was added and the resulting suspension stirred until all solids had dissolved. This solution was lyophilized to yield 3.77 g succinyl- β -Ala-Leu-Ala-Leu-Dox, 99% physical yield (89.06% HPLC purity by Method B).

25

Example 20

Synthesis of N-cap Succinyl form of Oligopeptide 38-Dnr-therapeutic agent [Suc- β -Ala-Leu-Ala-Leu-Dnr]

Piperidine (0.442 mL, 4.48 mmol) was added to a solution of Fmoc form of 30 Oligopeptide 38-Dnr therapeutic agent (100 mg, 0.089 mmol) in 5 mL of dry DMF. The reaction mixture was stirred for 5 minutes at room temperature and then cooled to -20 °C using dry ice/ acetone bath. Succinic anhydride (458 mg, 4.54 mmol) was added then to the cooled reaction mixture in one portion. The reaction was stirred

rapidly at -5 °C for 5 minutes then at room temperature for another 90 minutes. Anhydrous diethyl ether, 250 mL, was added to the reaction mixture and the resulting red precipitate was isolated on a medium glass frit. The filter cake was washed with two successive 50 mL portions of diethyl ether and dried under reduced pressure to 5 yield N-cap succinyl form of Oligopeptide 38-Dnr-therapeutic agent (80% yield, 88% HPLC purity by method B). The LC/MS gave a molecular weight of 995 (expected molecular weight 996).

Example 21

10 **Synthesis of N-cap Succinyl form of Oligopeptide 98-Daunorubicin Therapeutic Agent [Suc-Thi-Tyr-Gly-Leu-Dnr]**

To a solution of Fmoc form of Oligopeptide 98-Daunorubicin Therapeutic Agent (100 mg, 0.079 mmol) in 5 mL of dry DMF, piperidine (0.391 mL, 3.95 mmol), was added in one portion resulting in a color change from red to purple. The reaction 15 mixture was stirred for 5 minutes at room temperature and then cooled to -20° C using dry ice/ acetone bath. 407 mg (4.02 mmol) of succinic anhydride was then added to the cooled reaction mixture in one portion. The reaction was stirred rapidly at -5 °C for 5 minutes then at room temperature for another 90 minutes. Anhydrous diethyl ether, 200 mL, was added to the reaction mixture which resulted in the formation of a red precipitate. This precipitate was isolated on a medium glass frit, washed with 3 x 20 50 mL of diethyl ether and dried under reduced pressure to yield N-cap succinyl form of Oligopeptide 98-Dnr Therapeutic Agent (80% yield, 81% HPLC purity by method A). The LC/MS gave a molecular weight of 1141 (expected molecular weight 1142).

25 **Example 22**

Synthesis of Sodium Salt of N-cap Glutaryl form of Oligopeptide 38-Dox therapeutic agent [Gl-β-Ala-Leu-Ala-Leu-Dox]

Piperidine (436 µL, 4.413 mmol) was added to a solution of Fmoc form of Oligopeptide 38-Dox therapeutic agent (100 mg, 0.088 mmol) in DMF (4.5 mL). 30 After stirring for 5 minutes at room temperature, the reaction mixture was cooled to -5 °C and glutaric anhydride (624 mg, 5.472 mmol) was quickly added. The cold bath was removed as soon as the color changed and the mixture was stirred at room temperature for another 10 min. The DMF was removed by rotary evaporation and the

residue dissolved in chloroform (2.5 mL). Diethyl ether (14 mL) was added and the resulting precipitate filtered. The filter cake was washed with diethyl ether, air dried and then resuspended in water (14 mL). The sodium salt was formed by addition of 0.025 M NaOH (4 mL, 0.10 mmol) dropwise to the suspension until complete
5 dissolution of the solid. This solution was then lyophilized to give the sodium salt of glutaryl N-cap form of Oligopeptide 38-Dox therapeutic agent in 97% yield with an HPLC purity of 87% by method D.

Example 23

10 **“Urea method” for preparing the conjugate. i.e. Precursor for Enzyme Route
Coupling of Methyl Succinyl-N-cap form of Oligopeptide 38 and Doxorubicin**

Under dry nitrogen atmosphere 26.04g (52.0 mmol) methyl succinyl-N-cap form of Oligopeptide 38, 23.26g (40.2mmol) doxorubicin hydrochloride are suspended/dissolved in 800 mL dry, urea-saturated (~30% w/v) DMF and 14.8
15 19.948mL. 114.16 mmol DIEA. This mixture is cooled to 0-3°C over ~25 minutes. At this point 21.2 g (56.0 mmol) HATU added as a solution in ~100mL urea saturated DMF over 10 minutes (the volume of this solution should be kept minimal). The reaction mixture is stirred for 10 minutes at -2 to 2°C. and poured into 4000 mL ice cold brine, containing 2%v/v acetic acid over approximately five minutes with
20 vigorous stirring. The product is filtered off on a medium porosity fritted glass filter, washed generously with water and dried under reduced pressure. 43 g physical yield: 104.47 %, 93.45% pure by HPLC method B.

Example 24

25 **Synthesis of Methyl Succinyl-N-Cap form of Oligopeptide 38-Dox therapeutic agent [Methyl Succinyl- β Ala-Leu-Ala-Leu-Dox (MeOSuSLD)]**

In a round bottom flask (50 mL), N-cap methyl hemisuccinyl form of Oligopeptide 38 (0.25 g, 0.5 mmol) and doxorubicin (0.29 g, 0.5 mmol) was dissolved in anhydrous DMF (20 mL). After the mixture was stirred for 5 minutes, DIEA (0.17
30 mL, 1.0 mmol) followed by HBTU (0.19 g, 0.5 mmol) was added into the solution. The mixture was stirred at room temperature for 4 hrs. DMF was removed by a rotary evaporator and the residue was taken up in 4.0 mL 1:1 methylenechloride:methanol. To this solution, 40 mL of ether was slowly added while stirring. A red precipitate

was formed and collected by suction filtration. The solid was washed with ether (2x10 mL) and dried in a vacuum desiccator. 0.50 g of product (98%) was produced with purity of 96%.

5 Example 25

Hydrolysis of the Methyl Succinyl-N-cap form of Oligopeptide 38-Dox therapeutic agent via

Use of Cross Linked Enzyme

10 Methyl succinyl-N-cap form of Oligopeptide 38-Dox therapeutic agent (1.0 g, 0.975 mmol) and 100 mL DMF are placed in a 500 mL flask. The suspension is vigorously agitated with a magnetic stirrer. When the methyl succinyl-N-cap form of Oligopeptide 38-Dox therapeutic agent has completely dissolved, 400 mL deionized water is added and the resulting solution stirred
15 at 35 °C. A slurry of 1 g washed CLEC-PC (Altus Biologics) the immobilized enzyme was rinsed in three aliquots of deionized water then resuspended in 10 mL 20 % aqueous DMF prior to use.) suspended in 10 mL of 20% aqueous DMF is then added and the resulting suspension is stirred at 35°C with periodic HPLC monitoring. When all of the methyl succinyl-N-cap form of
20 Oligopeptide 38-Dox therapeutic agent has been consumed (~18 hours), the reaction mixture is filtered through a 0.45µM nylon membrane filter to remove the CLEC-PC enzyme. The CLEC-PC cake is washed with 3 X 10 mL methanol and the methanol washes are combined with the filtered reaction mixture. The filtered reaction mixture plus methanol washes is then
25 concentrated to a red gum on a rotary evaporator equipped with a high vacuum pump and a 30° C water bath. The red gum is then suspended in 50 mL deionized water at room temperature and rapidly stirred via mechanical stirrer. To this suspension a solution of 77.8 mg sodium bicarbonate (0.926 mmol, 0.95 eq.) in 100 mL deionized water is added over 2 minutes. The suspension
30 is stirred at room temperature 20 minutes. The reaction mixture is filtered through a 0.45 µM nylon membrane filter and lyophilized. 0.936 g sodium salt of succinyl-N-cap form of Oligopeptide 38-Dox therapeutic agent is isolated,

about 100% yield. 84 % pure HPLC method B. ¹H and ¹³C NMR spectra were recorded on 600 and 150 MHz spectrometers respectively and the electrospray MS, were consistent with the desired structure.

5 **Example 26**

Hydrolysis of the methyl succinyl-N-cap form of Oligopeptide 38-Dox therapeutic agent [Methyl Succinyl-β-Ala-Leu-Ala-Leu-dox]

Use of soluble enzyme

10 11.0 g (10.72 mmol) methyl succinyl-N-cap form of Oligopeptide 38-Dox therapeutic agent was suspended in 800 mL HPLC-grade water and homogenized for 60 minutes with an Ultraturrax T8 homogenizer to yield a finely divided suspension. This suspension was stirred (500 rpm) at 35° C and adjusted to pH=6.05 with aq. 76 mM NaHCO₃. 1.0 g C. Antarctica "B" lipase (Altus Biologics) was then added and
15 the reaction mixture stirred at 35° C for 48 hours. During the 48 hr reaction time, pH was maintained between 5.3 and 6.2 by periodic addition of 76 mM NaHCO₃ and the reaction was periodically monitored by HPLC. After 48 hours, the reaction was ca. 98% complete by HPLC. The reaction mixture was then adjusted to pH=7 with aq. 76 mM NaHCO₃ and filtered through a pad of Celite 521. The clarified reaction mixture
20 was then acidified to ca. pH 3 with 5 mL glacial acetic acid resulting in the formation of a gummy red precipitate. The precipitate was isolated by Celite 521 filtration, subsequent rinsing of the Celite pad with methanol, filtration of the methanol solution through a 10-20 µM fritted glass filter and rotary evaporation of the filtered solution to yield 7.31 g of gummy red product. This product was converted to the sodium salt
25 by dissolution in 70 mL 76 mM NaHCO₃ (0.95 eq.) and lyophilized to yield 7.30 g, 66.1% physical yield sodium salt of succinyl-N-cap form of Oligopeptide 38-Dox therapeutic agent, 84.5% pure by HPLC.

The product was identical the example above.

30 **Example 27**

Immobilized Candida Antarctica "B" Lipase Hydrolysis Methyl Succinyl-N-cap form of Oligopeptide 38-Dox therapeutic agent

30.0g *Candida Antarctica* "B" lipase (Altus Biologics) was dissolved in 300 mL water and dialyzed against 3 x 4 l of 50 mM aq. NaHCO₃ (pH=6.4). After dialysis, the volume of the dialyzed solution was ~300 mL. 360 mL of Pharmacia NHS-Activated Sepharose 4 Fast Flow was placed in a coarse glass fritted funnel and rinsed with 5 x 450 mL ice-cold 1 mM aq. HCl. The rinsed NHS-Activated Sepharose was then combined with the dialyzed enzyme solution. The resulting suspension was stirred at ambient temperature (ca. 22° C) for 2.0 hours. The sepharose/enzyme conjugate was then isolated on a coarse fritted glass filter and then stirred in 1000 mL 100 mM aq. TRIS (pH=7.45) for 15 minutes. This suspension was 5 filtered and incubated with another 1000mL 100 mM aqueous TRIS buffer (pH=7.45) at 4°C, overnight. The immobilized enzyme in the morning was filtered off and after washing with water placed into a 2000mL three necked, round bottomed flask. 43g methyl succinyl-N-cap form of Oligopeptide 38-Dox therapeutic agent added and the solids were suspended in 800mL deionized water. The flask is fitted with an overhead 10 stirrer, and a pH-stat set to keep the pH of the reaction mixture between 5.9-6.2 by controlling a syringe pump. The syringe pump was charged 0.1 M NaHCO₃. Progress of the reaction is followed by HPLC. After 6 days the immobilized enzyme was 15 filtered off and the liquid phase was lyophilized. The dry solids were then suspended in ~11 dry THF and filtered off. 42.66g, 98.34% physical yield, 93.43%(254nm), 20 94.43%(480nm) pure by HPLC by method B.

Example 28

Synthesis of the Lactate Salt of Oligopeptide 38-Dox Therapeutic Agent [β -Ala-Leu-Ala-Leu-Dox Lactate]

25 Piperidine (26 mL, 264 mmol) was added to a solution of Fmoc form of Oligopeptide 38-Dox therapeutic agent (6.00 g, 5.3 mmol) in DMF (265 mL). After stirring for 5 minutes at room temperature, the reaction mixture was placed in an ice-salt bath, and precooled (4°C) 10% lactate buffer pH 3 (600 mL) was immediately added. The aqueous solution was extracted with DCM (3x500 mL) and excess salts 30 were removed by solid phase extraction. C18 ODS-A silica gel (120 g) was conditioned (500 mL methanol, 2x500mL water) in a glass frit and loaded with the aqueous solution of crude product lactate salt. After washing with water (2x500 mL) and drying, the filter cake was dissolved in methanol. The methanol was evaporated

and the residue was dissolved in water. The resulting solution was lyophilized to give 3.54 g of lactate salt of Oligopeptide 38-Dox therapeutic agent (67% yield, HPLC purity method B: 89%).

5 **Example 29**

Synthesis of Succinyl-N-Cap Form of Oligopeptide 38-Dox Therapeutic Agent [succinyl- β -Ala-Leu-Ala-Leu-Dox] Starting From Lactate Salt of Oligopeptide 38-Dox Therapeutic Agent [β -Ala-Leu-Ala-Leu-Dox lactate].

DIEA (417 μ L, 2.40 mmol) was added to a solution of Lactate salt of
10 Oligopeptide 38-Dox therapeutic agent (1.200 g, 1.20 mmol) in DMF (35 mL). After stirring for 15 minutes at room temperature, succinic anhydride 97% (2) (0.144 g, 1.44 mmol) was added. The mixture was stirred for 2 h, and DMF was removed by rotary evaporation. The residue was dissolved in a mixture of CHCl₃/CH₃OH 4/1 (6 mL), and 200 mL of a mixture of Et₂O/hexane 1/1 were added. After the mixture was
15 stirred for 30 minutes, the precipitate was filtered on quantitative paper (Whatman 42), washed (Et₂O/hexane: 1/1) and air-dried. The filter cake was suspended in water (150 mL), and 1M NaOH (\pm 1.2 eq., 1.5 mL) was added dropwise until complete dissolution (pH=7.2). The solution was lyophilized to give 1.218 g of succinyl-N-cap form of Oligopeptide 38-Dox therapeutic agent (97% yield; HPLC purity method B:
20 80.2%.

Example 30

Synthesis of Succinyl-N-Cap Form of Oligopeptide 38-Dox Therapeutic Agent [succinyl- β -Ala-Leu-Ala-Leu-Dox] starting with Fmoc Form of Oligopeptide 38-

25 Dox Therapeutic Agent [Fmoc- β -Ala-Leu-Ala-Leu-Dox]

Piperidine (2180 μ L, 22.06 mmol) was added to a solution of Fmoc form of Oligopeptide 38-Dox therapeutic agent (0.50 g, 0.44 mmol) in DMF (21.5 mL). After stirring for 5 minutes at room temperature, the reaction mixture was quickly cooled to -5 °C and succinic anhydride (2.25 g, 22.51 mmol) was added immediately. The cold bath was removed as soon as the color changed and the mixture was stirred at room temperature for 10 minutes. The DMF was removed by rotary evaporation and the residue was dissolved in chloroform (12.5 mL). Diethylether (360 mL) was quickly added. A precipitate immediately appeared. The precipitate was filtered on Whatman
30

42 paper and washed with Et₂O. The solid was suspended in water (120 mL; pH=4.1) and 0.025M NaOH (20 mL, 0.53 mmol) was added dropwise until complete dissolution (pH=7.4). This solution was then lyophilized to give succinyl-N-cap form of Oligopeptide 38-Dox therapeutic agent in 89% yield and 91% HPLC purity by

5 Method D.

The invention therefore includes a prodrug compound, the compound comprising:

- (1) a therapeutic agent capable of entering a target cell,
- 10 (2) an oligopeptide having a formula (AA)_n-AA⁴-AA³-AA²-AA¹, wherein:
 - each AA independently represents any genetically encoded amino acid,
 - n is an integer from 0 to 12,
 - AA⁴ represents a non-genetically-encoded amino acid,
 - AA³ represents any amino acid,
 - 15 AA² represents any amino acid, and
 - AA¹ represents any amino acid,
- (3) a stabilizing group that hinders cleavage of said oligopeptide by enzymes present in whole blood, and
- (4) optionally, a linker group not cleavable by trypsin,
- 20 wherein the oligopeptide is directly linked to the stabilizing group at a first attachment site of the oligopeptide and AA¹ of the oligopeptide is directly linked to the therapeutic agent or indirectly linked through the linker group to the therapeutic agent at a second attachment site of the oligopeptide,
 - the compound being selectively cleaved by an enzyme associated with the
- 25 target cell.

Further, a method for decreasing toxicity of a therapeutic agent wherein the therapeutic agent is intended for administration to a patient, the method comprising: covalently forming a prodrug by linking an oligopeptide cleavable by trypsin to a stabilizing group at a first attachment site of the oligopeptide and directly or indirectly linking the therapeutic agent at a second attachment site of the oligopeptide, the prodrug being selectively cleaved by trypsin, whereby the prodrug provides for decreased toxicity of the therapeutic agent when administered to the patient.

The invention includes a method of making a prodrug compound agent comprising the following steps:

- (1) activating an Fmoc-protected oligopeptide with an activating agent in the presence of a therapeutic agent to make a Fmoc-protected oligopeptide therapeutic agent conjugate,
- (2) deprotecting the Fmoc-protected oligopeptide therapeutic agent by contacting it with a base,
- (3) reacting the oligopeptide therapeutic agent with a stabilizing group,
- 10 (4) neutralizing the stabilizing group-oligopeptide therapeutic agent conjugate with a pharmaceutically acceptable salt.

The invention also includes making a prodrug compound comprising the following steps:

- 15 (1) activating an alkyl ester-protected-stabilizing group oligopeptide with an activating agent in the presence of a therapeutic agent to make an alkylester-protected stabilizing group oligopeptide therapeutic agent conjugate,
- (2) deprotecting the alkyl ester-protected-stabilizing group oligopeptide therapeutic agent, and
- 20 (3) neutralizing the stabilizing group-oligopeptide therapeutic agent with a pharmaceutically acceptable salt.

Another aspect of the invention is a method of making a prodrug compound comprising the following steps:

- 25 (1) activating a trityl-protected oligopeptide with an activating agent in the presence of a therapeutic agent to make a trityl-protected oligopeptide therapeutic agent conjugate,
- (2) deprotecting the trityl-protected oligopeptide therapeutic agent conjugate under acidic conditions for 30-120 minutes at 0 to 25 °C,
- 30 (3) reacting the oligopeptide-therapeutic agent with a stabilizing group, and
- (4) neutralizing the stabilizing group-oligopeptide-therapeutic agent with a pharmaceutically acceptable salt.

Compositions of the invention include prodrugs made by all of the methods above.

5 All publications and patent applications mentioned in this specification are
herein incorporated by reference to the same extent as if each individual publication
or patent application was specifically and individually indicated to be incorporated by
reference.

10 The invention now being fully described, it will be apparent to one of ordinary
skill in the art that many changes and modifications can be made thereto without
departing from the spirit or scope of the appended claims.

WHAT IS CLAIMED IS:

1. A compound comprising:

(1) a therapeutic agent capable of entering a target cell,

5 (2) an oligopeptide having a formula $(AA)_n-AA^4-AA^3-AA^2-AA^1$, wherein:

each AA independently represents any genetically encoded amino acid,

n is an integer from 0 to 12,

AA^4 represents a non-genetically-encoded amino acid,

AA^3 represents any amino acid,

10 AA^2 represents any amino acid, and

AA^1 represents any amino acid,

(3) a stabilizing group that hinders cleavage of said oligopeptide by enzymes present in whole blood, and

(4) optionally, a linker group not cleavable by trypsin,

15 wherein the oligopeptide is directly linked to the stabilizing group at a first attachment site of the oligopeptide and AA^1 of the oligopeptide is directly linked to the therapeutic agent or indirectly linked through the linker group to the therapeutic agent at a second attachment site of the oligopeptide,

20 the compound being selectively cleaved by an enzyme associated with the target cell.

2. The compound of claim 1 wherein n is an integer from 0 to 8.

3. The compound of claim 1 wherein the target cell is a tumor or 25 inflammatory cell.

4. The compound of claim 1 wherein the enzyme associated with the target cell is trypsin.

30 5. The compound of claim 4 wherein the trypsin is present in the extracellular vicinity of the target cell for the therapeutic agent.

6. The compound of claim 4 wherein the trypsin cleaves the linkage between AA^3 and AA^2 of the oligopeptide.

7. The compound of claim 4 being a prodrug having an active portion, wherein the active portion of the prodrug is more permeable to the target cell after cleavage by the truase than prior to cleavage by the truase, the active portion
5 including at least the therapeutic agent.

8. The compound of claim 7 wherein the active portion of the prodrug consists of the therapeutic agent.

10 9. The compound of claim 7 wherein the active portion of the prodrug includes the therapeutic agent and at least a
the linker group.

10. The compound of claim 7 wherein the active portion of the prodrug
15 includes the therapeutic agent and AA¹ of the oligopeptide.

11. The compound of claim 9 further comprising AA² of the oligopeptide linked to AA¹.

20 12. The compound of claim 1 wherein the oligopeptide is selected from:
D-AlaThiβAlaβAlaLeuAlaLeu (SEQ ID NO: 1), ThiβAlaβAlaLeuAlaLeu (SEQ ID NO: 2), βAlaβAlaLeuAlaLeu (SEQ ID NO: 3), βAlaAlaAlaIle (SEQ ID NO: 4),
βAlaAlaAlaLeu (SEQ ID NO: 5), βAlaPheTyrLeu (SEQ ID NO: 6), βAlaPheThrPhe (SEQ ID NO: 7), βAlaPheGlyIle (SEQ ID NO: 8), βAlaPheGlyLeu (SEQ ID NO: 9),
25 βAlaPhePhePhe (SEQ ID NO: 10), βAlaPhePheIle (SEQ ID NO: 11),
βAlaPhePheLeu (SEQ ID NO: 12), βAlaPheAlaIle (SEQ ID NO: 13),
βAlaPheAlaLeu (SEQ ID NO: 14), ThiGlyAlaLeu (SEQ ID NO: 15), NalGlyAlaLeu (SEQ ID NO: 16), βAlaLeuTyrLeu (SEQ ID NO: 17), βAlaLeuThiLeu (SEQ ID NO: 18), βAlaLeuThrPhe (SEQ ID NO: 19), βAlaLeuThrIle (SEQ ID NO: 20),
30 βAlaLeuThrLeu (SEQ ID NO: 21), βAlaLeu (SerLeu (SEQ ID NO: 22),
βAlaLeuPyrLeu (SEQ ID NO: 23), βAlaLeuLeuLeu (SEQ ID NO: 24),
βAlaLeuGlyPhe (SEQ ID NO: 25), βAlaLeuGlyIle (SEQ ID NO: 26), ThiLeuGlyLeu (SEQ ID NO: 27), βAlaLeuGlyLeu (SEQ ID NO: 28), AibLeuGlyLeu (SEQ ID NO:

29). β AlaLeuPhelle (SEQ ID NO: 30), β AlaLeuPheLeu (SEQ ID NO: 31),
 β AlaLeuAibLeu (SEQ ID NO: 32), β AlaLeuAlaAla (SEQ ID NO: 33),
 β AlaLeuAla β Ala (SEQ ID NO: 34), β AlaLeuAlaPhe (SEQ ID NO: 35),
 β AlaLeuAlaGly (SEQ ID NO: 36), β AlaLeuAlaIle (SEQ ID NO: 37),
5 β AlaLeuAlaLeu (SEQ ID NO: 38), TicLeuAlaLeu (SEQ ID NO: 39), ThzLeuAlaLeu
(SEQ ID NO: 40), ThiLeuAlaLeu (SEQ ID NO: 41), NalLeuAlaLeu (SEQ ID NO:
42), NAALeuAlaLeu (SEQ ID NO: 43), D-LeuLeuAlaLeu (SEQ ID NO: 44), D-
AlaLeuAlaLeu (SEQ ID NO: 45), D-MetLeuAlaLeu (SEQ ID NO: 46),
APPLeuAlaLeu (SEQ ID NO: 47), AmbLeuAlaLeu (SEQ ID NO: 48),
10 β AlaLeuAlaNal (SEQ ID NO: 49), β AlaLeuAla (Ser (SEQ ID NO: 50),
 β AlaLeuAlaTyr (SEQ ID NO: 51), β AlaMetTyrPhe (SEQ ID NO: 52),
 β AlaMetTyrLeu (SEQ ID NO: 53), β AlaMetGlyIle (SEQ ID NO: 54), ThiMetGlyLeu
(SEQ ID NO: 55), β AlaMetPhePhe (SEQ ID NO: 56), β AlaMetPhelle (SEQ ID NO:
57), TicMetAlaLeu (SEQ ID NO: 58), NalMetAlaLeu (SEQ ID NO: 59),
15 NAAMetAlaLeu (SEQ ID NO: 60), β AlaMetAlaLeu (SEQ ID NO: 61),
APPMetAlaLeu (SEQ ID NO: 62), β AlaNleTyrIle (SEQ ID NO: 63), β AlaNleTyrLeu
(SEQ ID NO: 64), β AlaNleThrIle (SEQ ID NO: 65), β AlaNleThrLeu (SEQ ID NO:
66), β AlaNleGlyPhe (SEQ ID NO: 67), β AlaNleGlyIle (SEQ ID NO: 68),
 β AlaNleGlyLeu (SEQ ID NO: 69), β AlaNlePheIle (SEQ ID NO: 70), β AlaNleAlaIle
20 (SEQ ID NO: 71), β AlaNleAlaLeu (SEQ ID NO: 72), β AlaNleAlaPhe (SEQ ID NO:
73), β AlaNvaAlaLeu (SEQ ID NO: 74), β AlaPheTyrIle (SEQ ID NO: 75),
ThiProGlyLeu (SEQ ID NO: 76), ThiProAlaLeu (SEQ ID NO: 77), NalProAlaLeu
(SEQ ID NO: 78), β AlaProAlaLeu (SEQ ID NO: 79), β AlaPhe(Cl),AlaLeu (SEQ ID
NO: 80), β AlaPhe(NO₂),AlaIle (SEQ ID NO: 81), β AlaPhe(NO₂),AlaLeu (SEQ ID
NO: 82), β AlaPhgAlaLeu (SEQ ID NO: 83), β AlaPyrAlaLeu (SEQ ID NO: 84),
25 TicThrGlyLeu (SEQ ID NO: 85), β AlaThiGlyIle (SEQ ID NO: 86), β AlaThiAlaLeu
(SEQ ID NO: 87), β AlaTicAlaIle (SEQ ID NO: 88), β AlaTicAlaLeu (SEQ ID NO:
89), β AlaValAlaLeu (SEQ ID NO: 90), β AlaTrpAlaLeu (SEQ ID NO: 91),
 β AlaTyrTyrPhe (SEQ ID NO: 92), β AlaTyrTyrIle (SEQ ID NO: 93), β AlaTyrTyrLeu
30 (SEQ ID NO: 94), β AlaTyrThrLeu (SEQ ID NO: 95), β AlaTyrPheLeu (SEQ ID NO:
96), β AlaTyrGlyIle (SEQ ID NO: 97), ThiTyrGlyLeu (SEQ ID NO: 98),
 β AlaTyrGlyLeu (SEQ ID NO: 99), β AlaTyrPhelle (SEQ ID NO: 100), β AlaTyrAlaIle

(SEQ ID NO: 101), ThiTyrAlaLeu (SEQ ID NO: 102), and β AlaTyrAlaLeu (SEQ ID NO: 103).

13. The compound of claim 1 wherein AA¹ of the oligopeptide is selected
5 from Phenylalanine, Isoleucine, Alanine, Glycine, Tyrosine, 2-Naphthylalanine,
Serine, and β -Alanine.

14. The compound of claim 1 wherein AA² of the oligopeptide is selected
from 4Leucine, Tyrosine, Glycine, Serine, 3-Pyridylalanine, 2-Thienylalanine,
10 Aminoisobutyric Acid, Threonine, and Phenylalanine.

15. The compound of claim 1 wherein AA³ of the oligopeptide is selected
from Leucine, Tyrosine, Phenylalanine, *p*-Cl-Phenylalanine, *p*-Nitrophenylalanine,
Valine, Norleucine, Norvaline, Phenylglycine, Tryptophan, Tetrahydroisoquinoline-3-
15 carboxylic acid, 3-Pyridylalanine, Alanine, Glycine, Thienylalanine, Methionine,
Valine, and Proline.

16. The compound of claim 1 wherein AA⁴ is selected from β -Alanine,
Thiazolidine-4-carboxylic acid, 2-Thienylalanine, 2-Naphthylalanine, D-Alanine, D-
20 Leucine, D-Methionine, D-Phenylalanine, 3-Amino-3-phenylpropionic acid, γ -
Aminobutyric acid, 3-amino-4,4-diphenylbutyric acid, Tetrahydroisoquinoline-3-
carboxylic acid, 4-Aminomethylbenzoic acid, and Aminoisobutyric acid.

17. The compound of claim 1 wherein the stabilizing group is a
25 dicarboxylic or higher order carboxylic acid.

18. The compound of claim 1 wherein the stabilizing group is selected
from: Succinic acid, Diglycolic acid, Maleic acid, Polyethylene glycol, Pyroglutamic
acid, and Glutaric acid.

30

19. The compound of claim 1 wherein the stabilizing group is a non-
genetically encoded amino acid having four or more carbons.

20. The compound of claim 1 wherein the stabilizing group is one of aspartic acid linked to the oligopeptide at the β -carboxy group of the aspartic acid or glutamic acid linked to the oligopeptide at the γ -carboxy group of the glutamic acid.

5 21. The compound of claim 1 wherein the stabilizing group is negatively charged or neutral if administration to a patient of the therapeutic agent at a therapeutically effective dose results in aggregation of the therapeutic agent within the body of the patient.

10 22. The compound of claim 21 wherein the stabilizing group is negatively charged or neutral if the therapeutic agent aggregates when administered intravenously to the patient at a therapeutically effective dose.

15 23. The compound of claim 1 wherein the stabilizing group reduces interaction between the compound and endothelial cells that line blood vessels when administered to the patient.

24. The compound of claim 1 wherein the therapeutic agent is selected from Alkylating Agents, Antiproliferative agents, Tubulin Binding agents, Vinca Alkaloids, Enediynes, Podophyllotoxins or Podophyllotoxin derivatives, the Pteridine family of drugs, Taxanes, Anthracyclines, Dolastatins, Topoisomerase inhibitors, and *cis*-Platinums.

25 25. The compound of claim 1 wherein the therapeutic agent is selected from Doxorubicin, Daunorubicin, Vinblastine, Vincristine, Calicheamicin, Etoposide, Etoposide phosphate, CC-1065, Duocarmycin, KW-2189, Methotrexate, Methotrexate, Aminopterin, Dichloromethotrexate, Docetaxel, Paclitaxel, Epithiophane, Combretastatin, Combretastatin A₄ Phosphate, Dolastatin 10, Dolastatin 11, Dolastatin 15, Topotecan, Camptothecin, Mitomycin C, Porfiromycin, 5-Fluorouracil, 30 6-Mercaptopurine, Fludarabine, Tamoxifen, Cytosine arabinoside, Adenosine arabinoside, Colchicine, Carboplatin, Mitomycin C, Bleomycin, Melphalan, and derivatives and analogs thereof.

26. The compound of claim 1 wherein the therapeutic agent has an intracellular active site.

27. The compound of claim 1 wherein AA¹ of the oligopeptide is directly linked to the therapeutic agent.

28. The compound of claim 1 wherein AA¹ of the oligopeptide sequence is indirectly linked to the therapeutic agent at the second attachment site of the oligopeptide via a linker group, the linker group selected from amino caproic acid, hydrazide group, an ester group, an ether group, and a sulphydryl group.

29. A pharmaceutical composition comprising

(1) a compound including

(a) a therapeutic agent capable of entering a target cell,

(b) an oligopeptide having a formula (AA)_n-AA⁴-AA³-AA²-AA¹, wherein:

each AA independently represents any genetically encoded amino acid,

n is an integer from 0 to 12,

AA⁴ represents a non-genetically-encoded amino acid,

AA³ represents any amino acid,

AA² represents any amino acid, and

AA¹ represents any amino acid,

(c) a stabilizing group that hinders cleavage of said oligopeptide by enzymes present in whole blood, and

(d) optionally, a linker group not cleavable by trypsin,

wherein the oligopeptide is directly linked to the stabilizing group at a first attachment site of the oligopeptide and AA¹ of the oligopeptide is directly linked to the therapeutic agent or indirectly linked through the linker group to the therapeutic agent at a second attachment site of the oligopeptide,

the compound being selectively cleaved by an enzyme associated with the target cell, and

(2) a pharmaceutically acceptable carrier.

30. A method for decreasing toxicity of a therapeutic agent wherein the therapeutic agent is intended for administration to a patient, the method comprising:
covalently forming a prodrug by linking an oligopeptide cleavable by trouse to a stabilizing group at a first attachment site of the oligopeptide and directly or
5 indirectly linking the therapeutic agent at a second attachment site of the oligopeptide, the prodrug being selectively cleaved by trouse, whereby the prodrug provides for decreased toxicity of the therapeutic agent when administered to the patient.

31. The method of claim 30 wherein the prodrug allows for administration
10 of an increased dosage of the therapeutic agent to the patient relative to the dosage of the therapeutic agent without a prodrug linkage.

32. A prodrug formed by the method of 30.

15 33. A method of making a prodrug compound agent comprising the following steps:

(1) activating an Fmoc-protected oligopeptide with an activating agent in the presence of a therapeutic agent to make a Fmoc-protected oligopeptide therapeutic agent conjugate,
20 (2) deprotecting the Fmoc-protected oligopeptide therapeutic agent by contacting it with a base,
(3) reacting the oligopeptide therapeutic agent with a stabilizing group,
(4) neutralizing the stabilizing group-oligopeptide therapeutic agent conjugate with a pharmaceutically acceptable salt.

25 34. The method of claim 33, wherein the oligopeptide is the Fmoc-form of β -AlaLeuAlaLeu.

30 35. The method of claim 33, wherein the therapeutic agent is an anthracycline.

36. The method of claim 33, wherein the step of activating an Fmoc-protected oligopeptide with an activating agent further comprises selecting an

activating agent from HATU, HBTU, DCC, DIC, DCC+HOBr, EDC, OSu, and PyBOP.

37. The method of claim 33, wherein the step of activating an Fmoc-
5 protected oligopeptide uses the HATU or HBTU activating agent.

38. The method of claim 33, wherein the step of deprotecting the Fmoc-protected oligopeptide therapeutic agent further comprises selecting from piperidine, DBU, DBN, DBO, triethylamine, or NaOH.

10

39. The method of claim 33, wherein the step of activating the oligopeptide to the therapeutic agent further comprises using a solvent that is saturated with a chaotropic agent.

15

40. The method of claim 33, wherein the step of coupling the oligopeptide to the therapeutic agent further comprises using the solvent DMF saturated with the chaotropic agent, urea.

41. The method of claim 33, wherein the step of reacting the oligopeptide
20 therapeutic agent with a stabilizing group uses an anhydride or an activated ester of the stabilizing group.

42. A composition made by the method of claim 33.

25

43. A method of making a prodrug compound comprising the following steps:

30

- (1) activating an alkyl ester-protected-stabilizing group oligopeptide with an activating agent in the presence of a therapeutic agent to make an alkylester-protected stabilizing group oligopeptide therapeutic agent conjugate,
- (2) deprotecting the alkyl ester-protected-stabilizing group oligopeptide therapeutic agent, and
- (3) neutralizing the stabilizing group-oligopeptide therapeutic agent with a pharmaceutically acceptable salt.

44. The method of claim 43, wherein the alkyl ester-protected stabilizing group oligopeptide is protected by methyl or ethyl ester group.

45. The method of claim 43, wherein the oligopeptide is methylsuccinyl protected β -AlaLeuAlaLeu.

46. The method of claim 43, wherein the therapeutic agent is an anthracycline.

10 47. The method of claim 43, wherein the step of activating the alkyl ester-protected stabilizing group oligopeptide with an activating agent comprises selecting from HATU, HBTU, DCC, DIC, DCC+HOBt, EDC, OSu, and PyBOP.

15 48. The method of claim 43, wherein the step of deprotecting the alkyl ester-protected stabilizing group oligopeptide therapeutic agent conjugate further comprises deprotecting with an enzyme selected from esterases, CHIRO CLEC-PCTM, pig liver esterase, *Candida Antarctica* B lipase, and Sepharose-immobilized *Candida Antarctica* B lipase.

20 49. The method of claim 43, wherein the step of activating the alkyl ester-protected stabilizing group oligopeptide further comprises using a solvent that is saturated with a chaotropic agent.

25 50. The method of claim 43, wherein the step of activating the oligopeptide to the therapeutic agent further comprises using the solvent DMF saturated with the chaotropic agent, urea.

30 51. The method of claim 43, wherein the concentration of the alkyl ester-protected stabilizing group oligopeptide therapeutic agent is 1-25 % in the conjugation solvent and the deprotection solvent.

52. The method of claim 43, wherein the alkyl ester-protected group is alkyl hemisuccinyl ester.

53. The method of claim 43, wherein the step of deprotecting the alkyl ester-protected stabilizing group oligopeptide therapeutic agent further comprises deprotecting with $Pd(P(Ph_3)_4)$.

5

54. A composition made by the method of claim 43.

55. A method of making a prodrug compound comprising the following steps:

10 (1) activating a trityl-protected oligopeptide with an activating agent in the presence of a therapeutic agent to make a trityl-protected oligopeptide therapeutic agent conjugate,

(2) deprotecting the trityl-protected oligopeptide therapeutic agent conjugate under acidic conditions for 30-120 minutes at 0 to 25 °C,

15 (3) reacting the oligopeptide-therapeutic agent with a stabilizing group, and

(4) neutralizing the stabilizing group-oligopeptide-therapeutic agent with a pharmaceutically acceptable salt.

56. The method of claim 55, wherein the oligopeptide is the trityl form of
20 β -AlaLeuAlaLeu.

57. The method of claim 55, wherein the therapeutic agent is an anthracycline.

25 58. The method of claim 55, wherein the step of activating the trityl-protected oligopeptide with an activating agent further comprises selecting an active agent from HATU, HBTU, DCC, DIC, DCC+HOBt, EDC, OSu, and PyBOP.

30 59. The method of claim 55, wherein the step of activating the oligopeptide to the therapeutic agent further comprises using a solvent that is saturated with a chaotropic agent.

60. The method of claim 59, wherein the step of activating further comprises solvent DMF saturated with urea as the chaotropic agent.

5 61. The method of claim 55, wherein the step of reacting the oligopeptide therapeutic agent with a stabilizing group further comprises use of an anhydride or an activated ester of the stabilizing group.

10 62. The method of claim 61, wherein the step of reacting the oligopeptide therapeutic agent with a stabilizing group further comprises using a succinic or glutaric anhydride or respective methyl hemiester of succinic acid or glutaric acid as the stabilizing group.

15 63. The method of claim 55, wherein the step of neutralizing the stabilizing group-oligopeptide-therapeutic agent further comprises using a sodium bicarbonate conjugate as the pharmaceutically acceptable salt.

64. The method of claim 55, wherein the concentration of trityl-protected oligopeptide therapeutic agent conjugate is 1-25 % in the conjugation solvent.

20 65. A composition made by the method of claim 55.

66. A compound selected from the following: Suc- β Ala-Leu-Ala-Leu-Dox, Suc- β Ala-Leu-Ala-Leu-Dnr, and Glutaryl- β Ala-Leu-Ala-Leu-Dox.

25 67. A compound selected from the following:
 β Ala -Leu-Ala-Leu-Dox

Trityl- β Ala -Leu-Ala-Leu-Dox

Diphenylmethyl- β Ala -Leu-Ala-Leu-Dox

Benzylloxycarbonyl- β Ala -Leu-Ala-Leu-Dox

30 Fmoc- β Ala -Leu-Ala-Leu-OBn

β Ala -Leu-Ala-Leu-OBn

Methyl-succinyl- β Ala -Leu-Ala-Leu-OBn

Methyl-succinyl- β Ala -Leu-Ala-Leu

Fmoc- β Ala -Leu-Ala-Leu

Fmoc-Thi-Tyr-Gly-Leu
Fmoc- β Ala -Leu-Ala-Leu-Dnr
Fmoc-Thi-Tyr-Gly-Leu-Dnr
Suc-Thi-Tyr-Gly-Leu-Dnr

5 Gl- β Ala -Leu-Ala-Leu-Dox
 β Ala -Leu-Ala-Leu-Dox Lactate
Allyl-succinyl- β Ala -Leu-Ala-Leu-Dox
Suc- β Ala -Leu-Ala-Leu
Methyl esters of Suc- β Ala -Leu-Ala-Leu

10 Fmoc- β Ala -Leu-Ala-Leu-Dox
Methyl-succinyl- β Ala -Leu-Ala-Leu-Dox, and
Allyl-hemi succinate.

1/17

Symbol	Name	Structure
Aca	6-Aminocaproic Acid	$\text{H}_2\text{N}-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{CH}_2-\text{COOH}$
Aib	Aminoisobutyric Acid	$\text{H}_2\text{N}-\text{CH}(\text{CH}_3)-\text{CH}(\text{CH}_3)-\text{COOH}$
Amb	4-(Aminomethyl)benzoic Acid	$\text{H}_2\text{N}-\text{CH}_2-\text{C}_6\text{H}_4-\text{COOH}$
APP	3-Amino-3-phenylpropionic Acid	$\text{C}_6\text{H}_5-\text{CH}(\text{NH}_2)-\text{CH}_2-\text{COOH}$
Dg	Diglycolic Acid	$\text{HO}-\text{CH}_2-\text{CO}-\text{O}-\text{CH}_2-\text{CO}-\text{OH}$

FIG. 1A

2/17

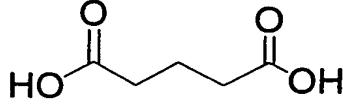
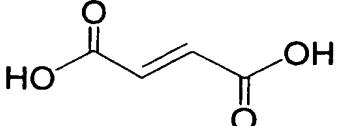
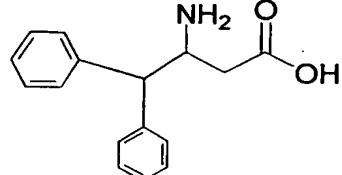
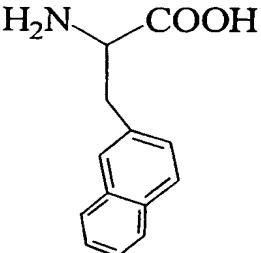
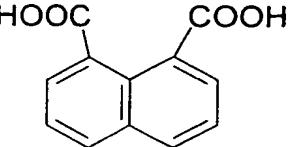
Symbol	Name	Structure
Gl	Glutaric Acid	
Mal	Maleic Acid	
NAA	3-Amino-4,4-diphenylbutyric Acid	
Nal	2-Naphthylalanine	
Naph	1,8-Naphthalene dicarboxylic Acid	

FIG. 1B

3/17

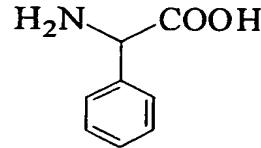
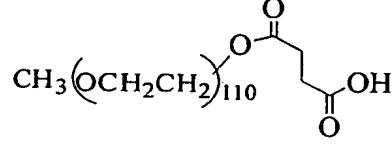
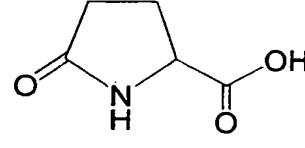
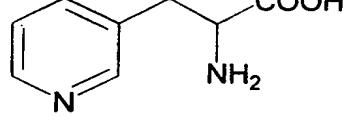
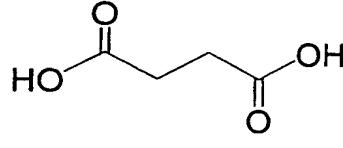
Symbol	Name	Structure
Phg	Phenylglycine	
PEG	Polyethylene Glycol ₅₀₀₀ Hemisuccinyl Ester	
Pyg	Pyroglutamic Acid	
Pyr	3-Pyridylalanine	
Suc	Succinic Acid	

FIG. 1C

4/17

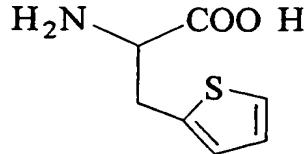
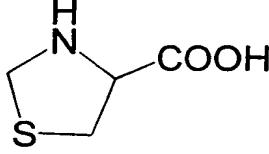
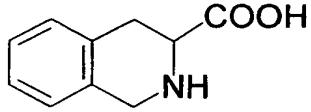
Symbol	Name	Structure
Thi	2-Thienylalanine	
Thz	3-Thioproline or Thiazolidine-4-carboxylic Acid	
Tic	Tetrahydroisoquinoline-3-carboxylic Acid	

FIG. 1D

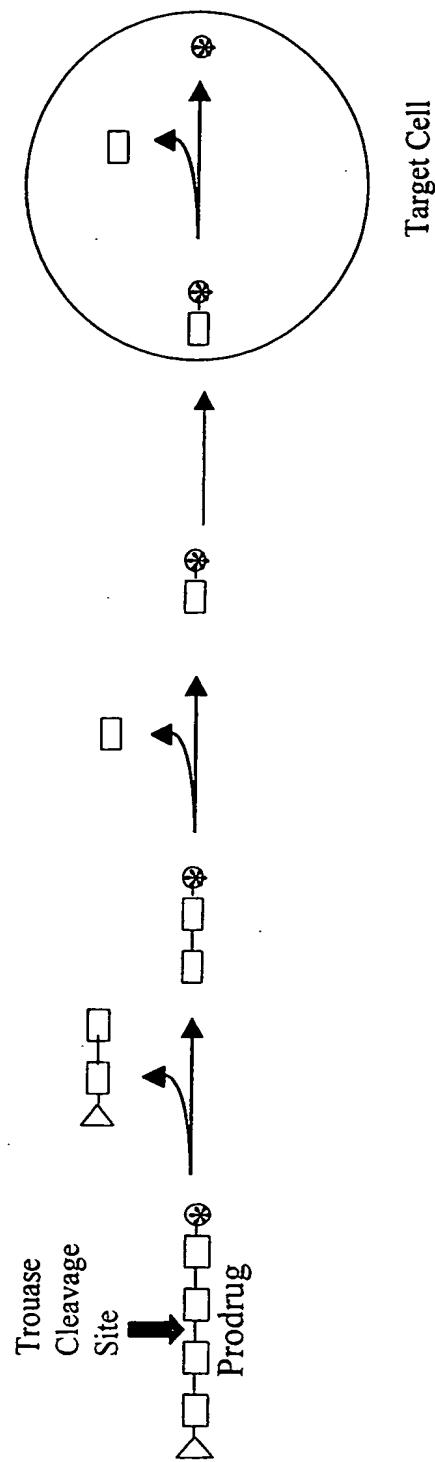


FIG. 2

6/17

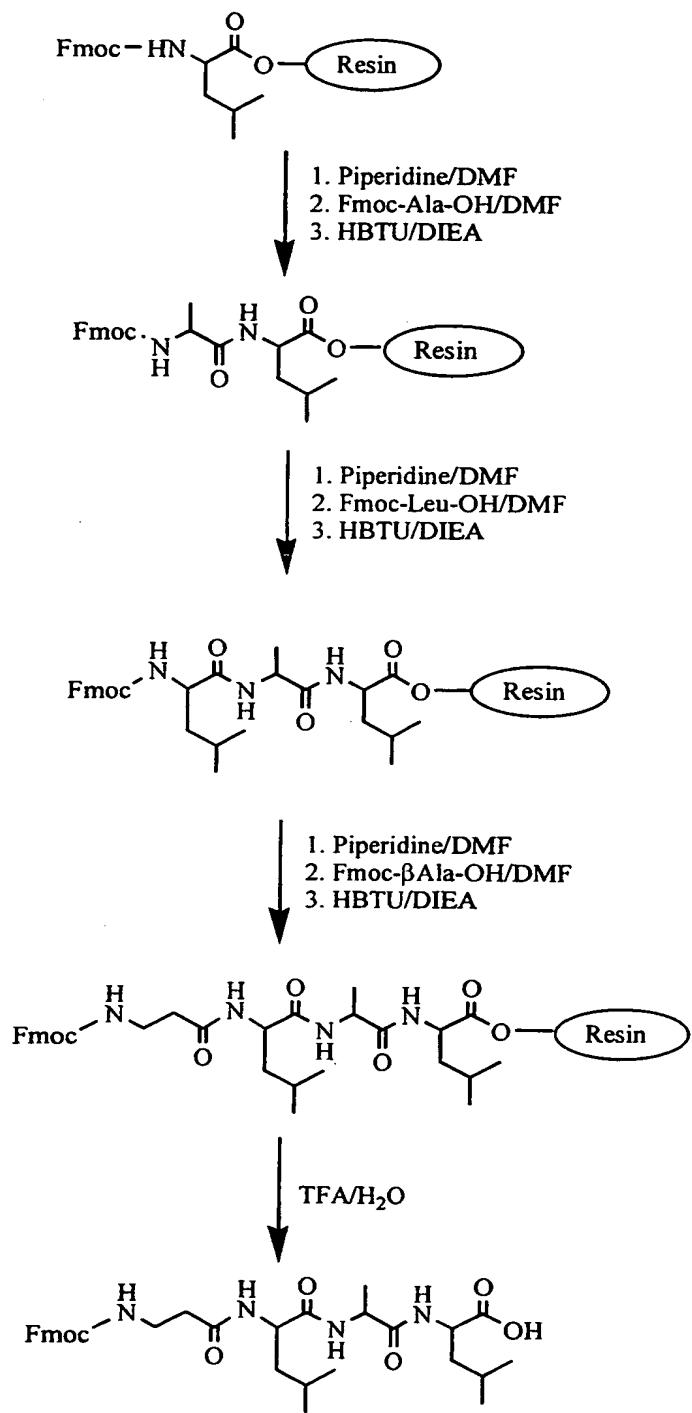


FIG. 3

7/17

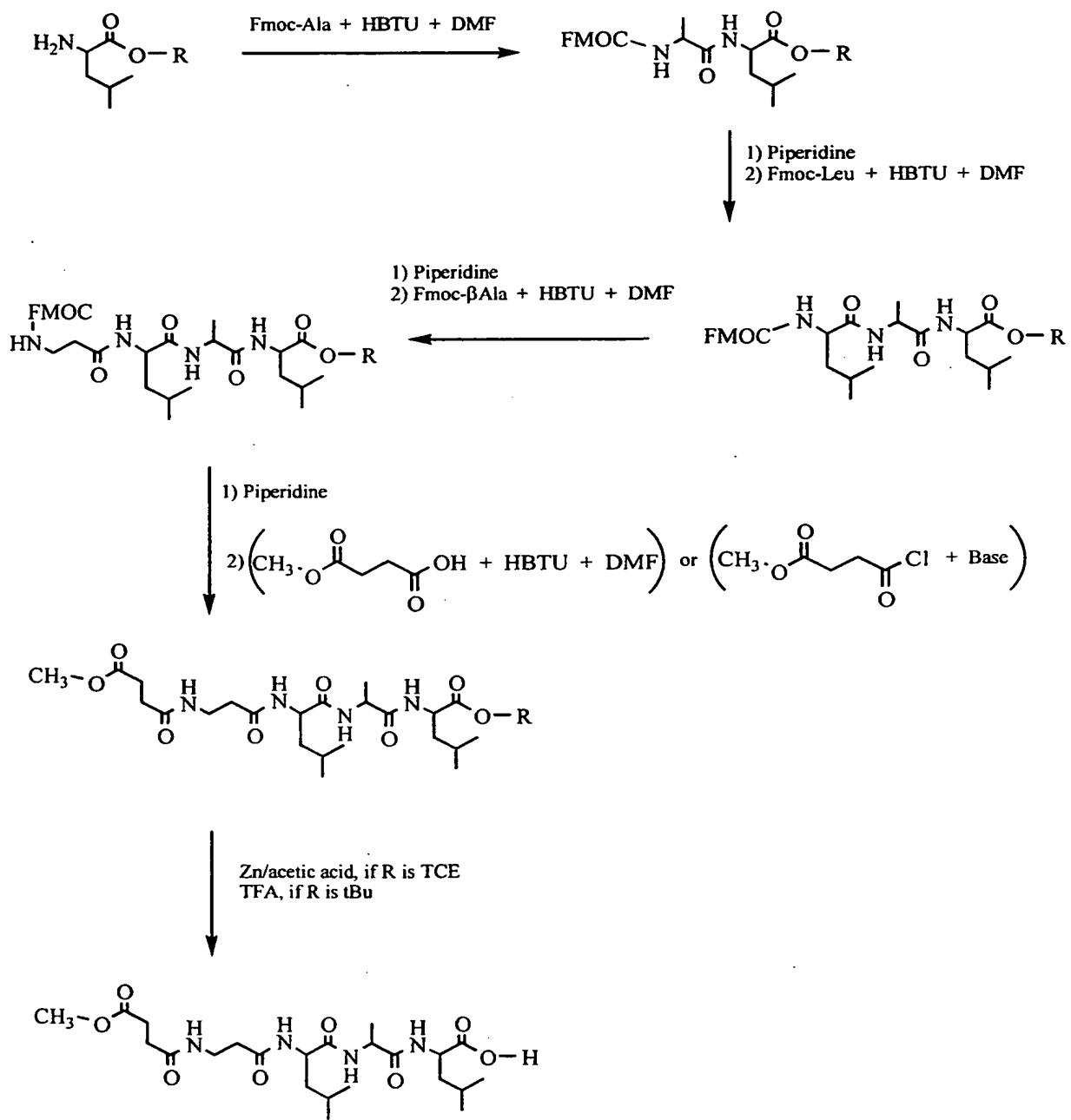


FIG. 4

8/17

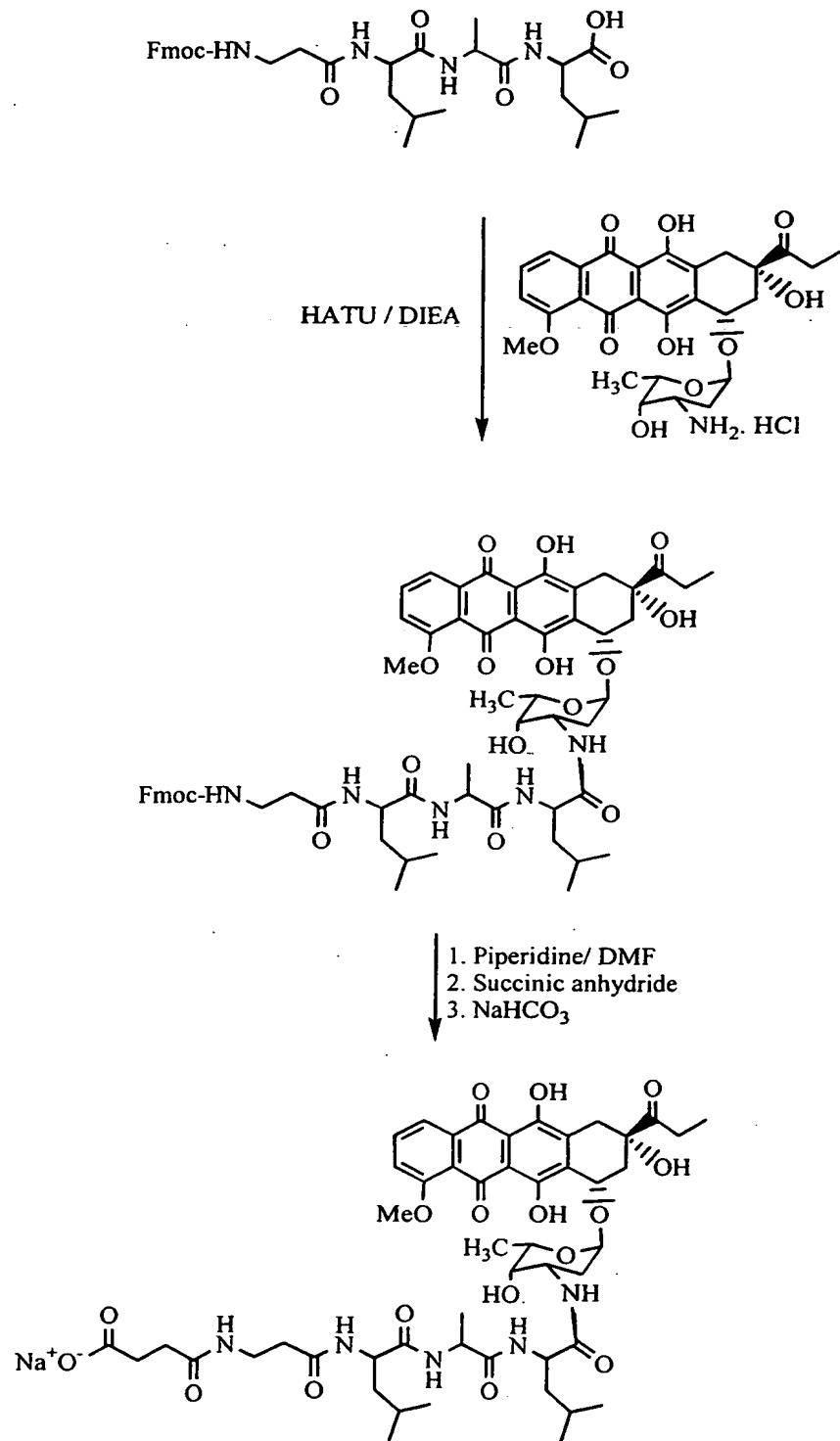


FIG. 5

9/17

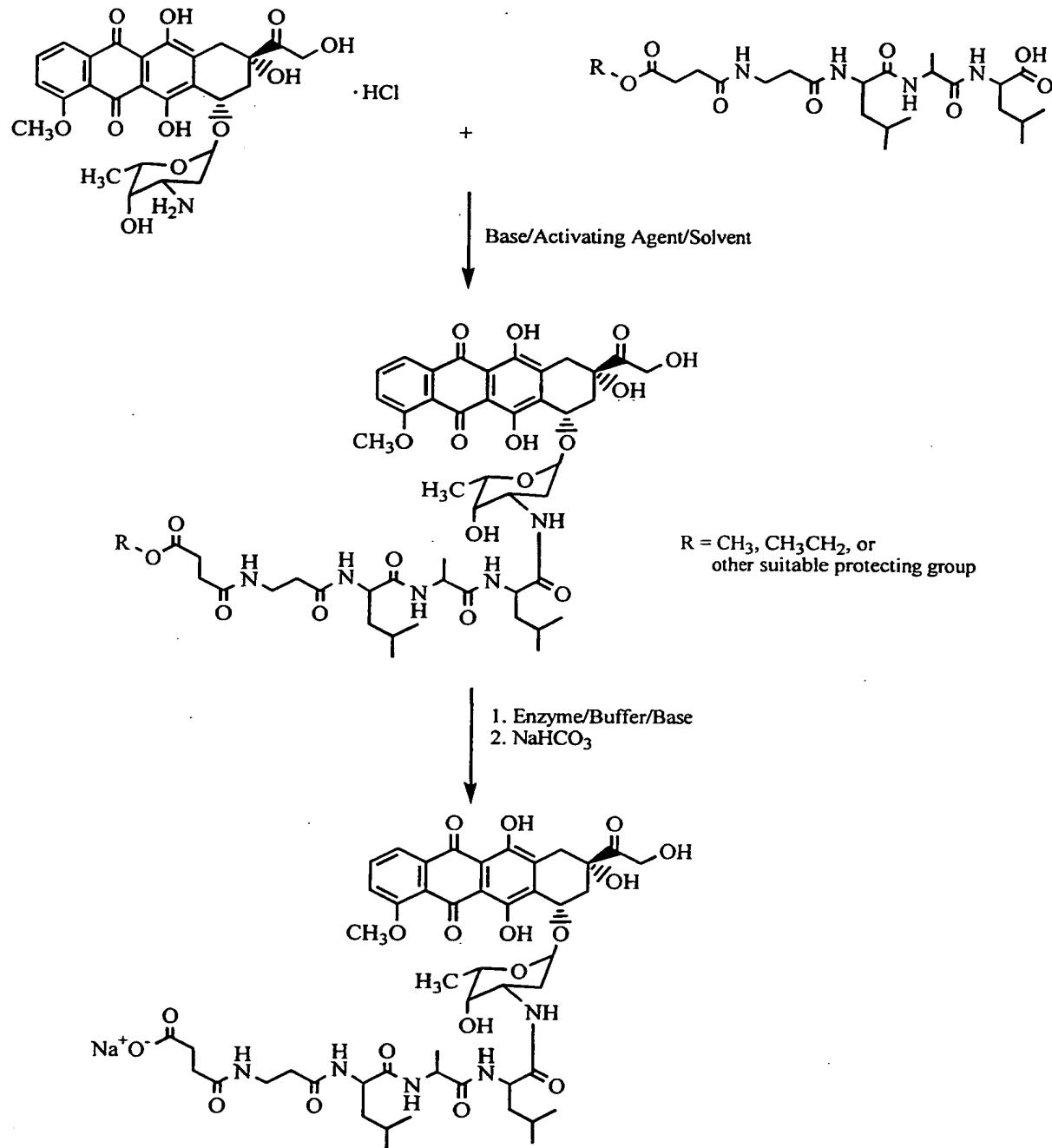


FIG.6

10/17

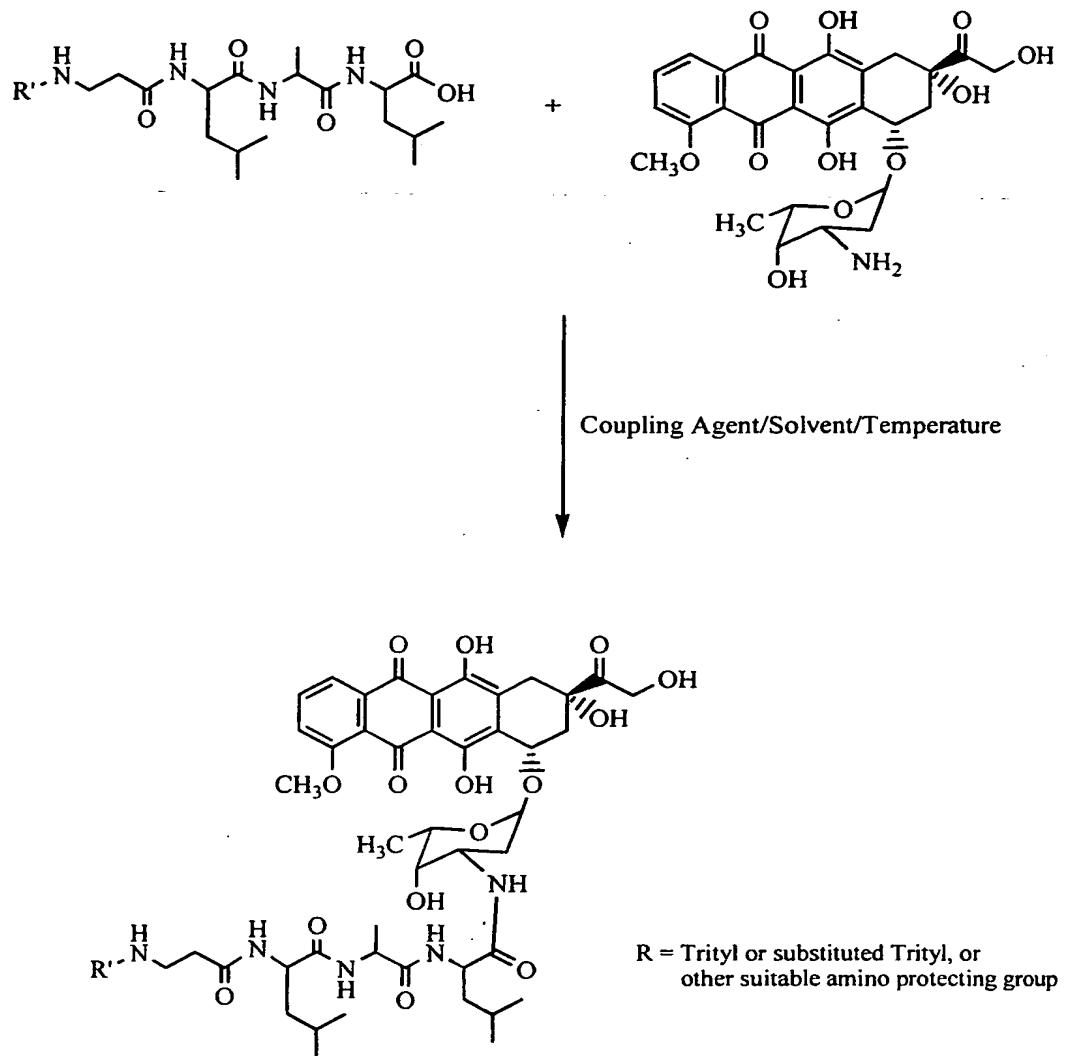


FIG. 7

11/17

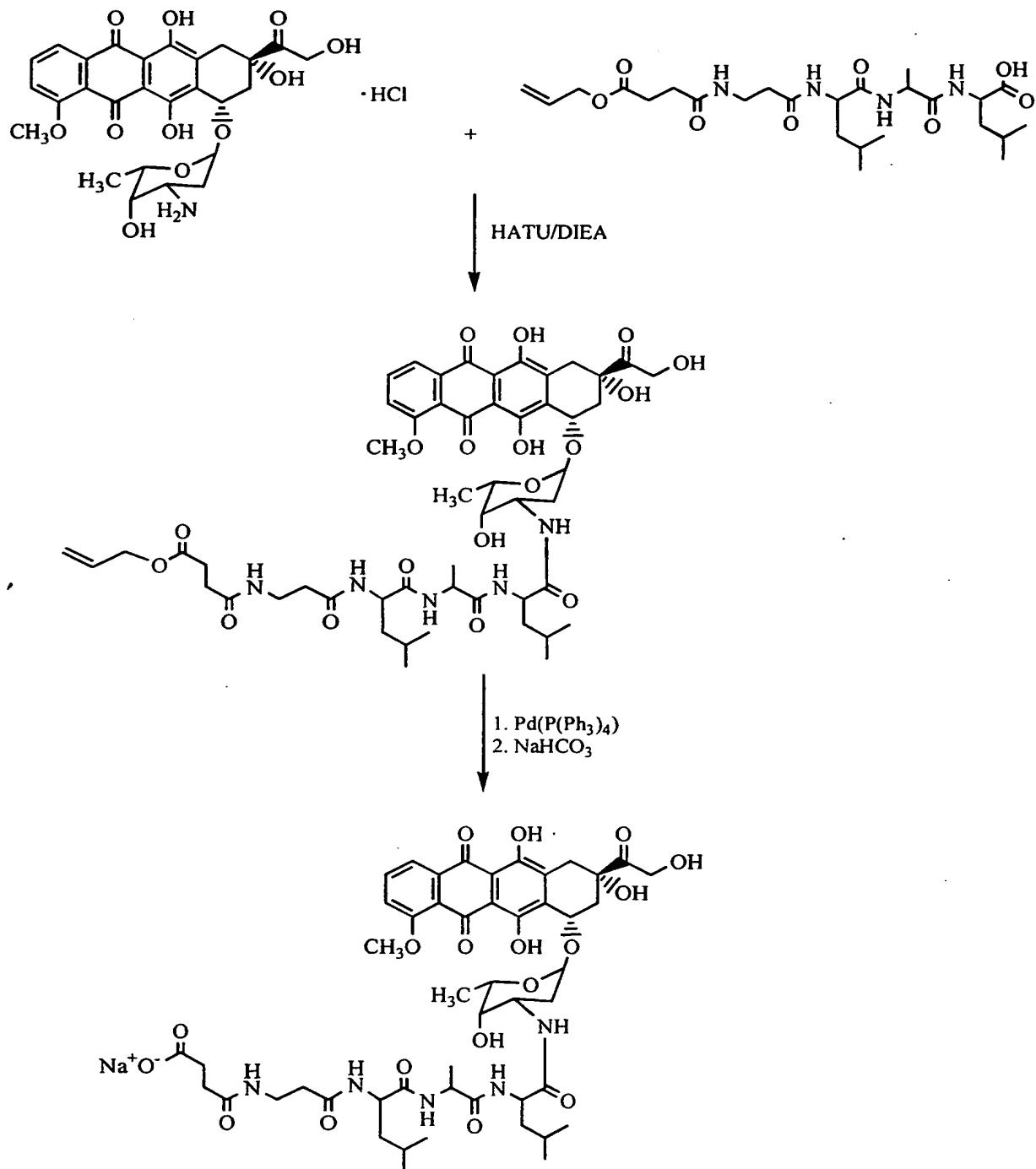


FIG. 8

12/17

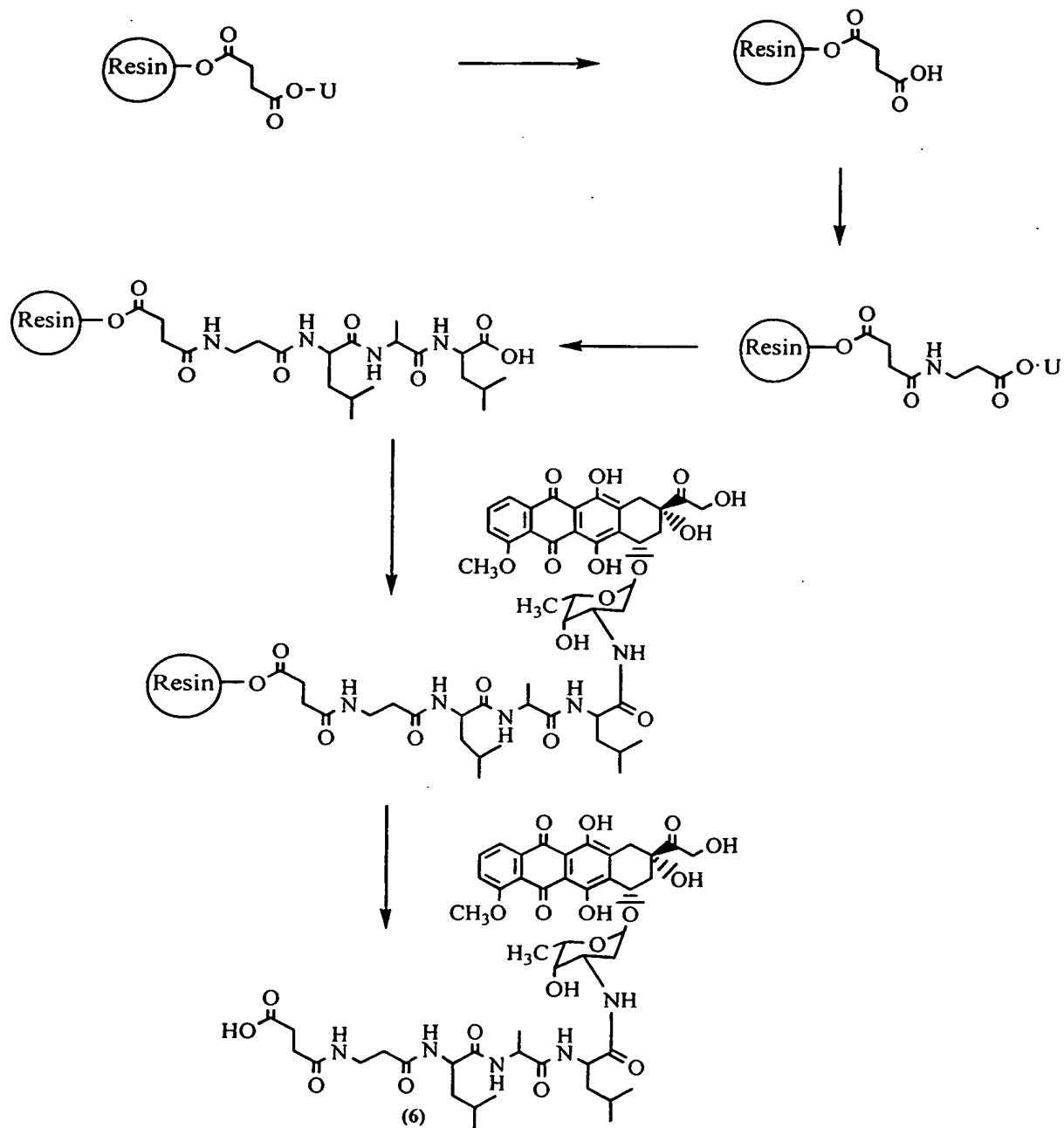


FIG. 9

13/17

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2	Ø	Thi	βAla	βAla	Leu	Ala	Leu	SEQ ID NO: 2
3	Ø	Ø	βAla	βAla	Leu	Ala	Leu	SEQ ID NO: 3
4	Ø	Ø	Ø	βAla	Ala	Ala	Ile	SEQ ID NO: 4
5	Ø	Ø	Ø	βAla	Ala	Ala	Leu	SEQ ID NO: 5
6	Ø	Ø	Ø	βAla	Phe	Tyr	Leu	SEQ ID NO: 6
7	Ø	Ø	Ø	βAla	Phe	Thr	Phe	SEQ ID NO: 7
8	Ø	Ø	Ø	βAla	Phe	Gly	Ile	SEQ ID NO: 8
9	Ø	Ø	Ø	βAla	Phe	Gly	Leu	SEQ ID NO: 9
10	Ø	Ø	Ø	βAla	Phe	Phe	Phe	SEQ ID NO: 10
11	Ø	Ø	Ø	βAla	Phe	Phe	Ile	SEQ ID NO: 11
12	Ø	Ø	Ø	βAla	Phe	Phe	Leu	SEQ ID NO: 12
13	Ø	Ø	Ø	βAla	Phe	Ala	Ile	SEQ ID NO: 13
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25	Ø	Ø	Ø	βAla	Leu	Gly	Phe	SEQ ID NO: 25
26	Ø	Ø	Ø	βAla	Leu	Gly	Ile	SEQ ID NO: 26
27	Ø	Ø	Ø	Thi	Leu	Gly	Leu	SEQ ID NO: 27
28	Ø	Ø	Ø	βAla	Leu	Gly	Leu	SEQ ID NO: 28
29	Ø	Ø	Ø	Aib	Leu	Gly	Leu	SEQ ID NO: 29
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36	Ø	Ø	Ø	βAla	Leu	Ala	Gly	SEQ ID NO: 36
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38	Ø	Ø	Ø	βAla	Leu	Ala	Leu	SEQ ID NO: 38
39	Ø	Ø	Ø	Tic	Leu	Ala	Leu	SEQ ID NO: 39
40	Ø	Ø	Ø	Thz	Leu	Ala	Leu	SEQ ID NO: 40

FIG. 10A

14/17

No:	(AA ¹)	(AA ⁶)	(AA ⁵)	(AA ⁴)	(AA ³)	(AA ²)	(AA ¹)	SEQ ID NO:
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45	Ø	Ø	Ø	D-Ala	Leu	Ala	Leu	SEQ ID NO: 45
46	Ø	Ø	Ø	D-Met	Leu	Ala	Leu	SEQ ID NO: 46
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48	Ø	Ø	Ø	Amb	Leu	Ala	Leu	SEQ ID NO: 48
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53	Ø	Ø	Ø	βAla	Met	Tyr	Leu	SEQ ID NO: 53
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55	Ø	Ø	Ø	Thi	Met	Gly	Leu	SEQ ID NO: 55
56	Ø	Ø	Ø	βAla	Met	Phe	Phe	SEQ ID NO: 56
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78	Ø	Ø	Ø	Nal	Pro	Ala	Leu	SEQ ID NO: 78
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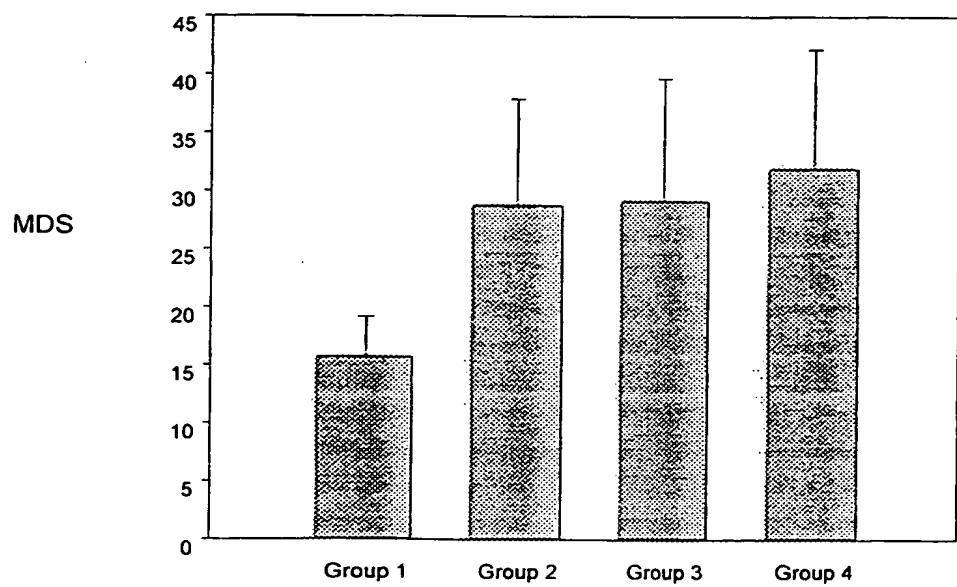
FIG. 10B

15/17

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84	Ø	Ø	Ø	βAla	Pyr	Ala	Leu	SEQ ID NO: 84
85	Ø	Ø	Ø	Tic	Thr	Gly	Leu	SEQ ID NO: 85
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89	Ø	Ø	Ø	βAla	Tic	Ala	Leu	SEQ ID NO: 89
90	Ø	Ø	Ø	βAla	Val	Ala	Leu	SEQ ID NO: 90
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92	Ø	Ø	Ø	βAla	Tyr	Tyr	Phe	SEQ ID NO: 92
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98	Ø	Ø	Ø	Thi	Tyr	Gly	Leu	SEQ ID NO: 98
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101	Ø	Ø	Ø	βAla	Tyr	Ala	Ile	SEQ ID NO: 101
102	Ø	Ø	Ø	Thi	Tyr	Ala	Leu	SEQ ID NO: 102
103	Ø	Ø	Ø	βAla	Tyr	Ala	Leu	SEQ ID NO: 103

FIG. 10C

16/17

**FIG. 11**

17/17

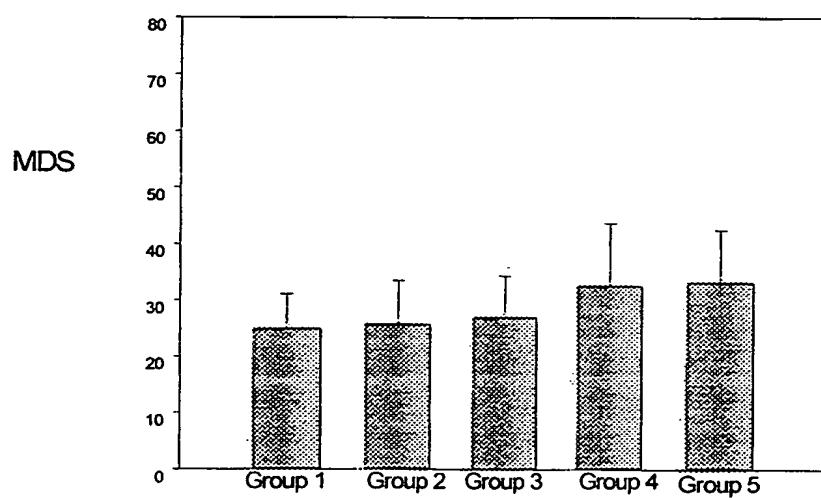


FIG. 12

SEQUENCE LISTING

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<140> not yet available

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<150> 60/119,312

<151> 1999-02-08

<150> 60/111,793

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